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UNIVERSITY OF ALBERTA

CHARACTERIZATION OF *TOLYPOCLADIUM NIVEUM* CYCLOSPORIN MUTANTS

BY

ELIZABETH MUKANYANGA MUZUNGAILE ©

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

SPRING, 1992

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CHARACTERIZATION OF *TOLYPOCLADIUM NIVEUM* CYCLOSPORIN MUTANTS submitted by ELIZABETH MUKANYANGA MUZUNGAILE in partial fulfillment of the requirements for the degree of Master of Science.

M A Pickard
Supervisor Dr. M.A. Pickard

H Anwar
Dr. H. Anwar

Dr. W. Ayer

Kenneth L. Roy
Dr. K.L. Roy

Date: 29 November 1991

DEDICATION

To my husband Webster, and our children Simba and Farai, for their love.

ABSTRACT

The use of UV-light and epichlorohydrin to isolate mutants capable of producing high levels of cyclosporin in the presence of 4-aza-DL-leucine was examined. Using UV-light, mutant UV-98, capable of producing 47% more cyclosporin was isolated from *T. niveum* UAMH 4002. A second round of mutagenesis involved the treatment of mutant UV-98 with epichlorohydrin and led to the isolation of mutant EPI-74 .

Growth of these mutants in medium containing different carbon sources showed that the carbon source supporting the highest amount of cyclosporin yield was strain specific. Increasing the concentration of carbon source led to an increase in biomass but not cyclosporin production. The use of ammonium sulfate as a nitrogen source proved unsuitable in one mutant strain while Delcase, a complex nitrogen source, increased cyclosporin production in wild type *T. niveum* UAMH 2472 (139% more than Vitamin Assay Casamino Acids) but not in mutant B-5 (UV derived mutant of UAMH 2472, Fode, 1990). Supplementation of the growth medium with constituent amino acids demonstrated that production towards certain forms of cyclosporin could be directed but did not always lead to increased titres except in the case of L-valine. Addition of L-asparagine, a non-constituent amino acid showed that cyclosporin synthesis could be adversely affected by molecules which are not part of its normal structure. Examination of the relationship between growth and cyclosporin production showed that the high levels of cyclosporin observed in mutant UV-98 compared to wild type UAMH 4002 were due to high rate of production by the former.

Four proteins which may be involved in the synthesis of cyclosporin were obtained using two different purification procedures. Their molecular weights were estimated to be 5.0 x10^5, 4.7 x10^5, 4.4 x 10^5 and 4.1 x10^5 daltons for proteins A, B, C, and D respectively.

Using contour-clamped homogeneous electric field gel electrophoresis, *T. niveum* chromosomes were separated into four bands with molecular sizes ranging from 1.2 to 5.6 megabases. The total genome size was determined to be between 20.1 and 21.3 Mb.

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1. INTRODUCTION:

1.1 CYCLOSPORIN:

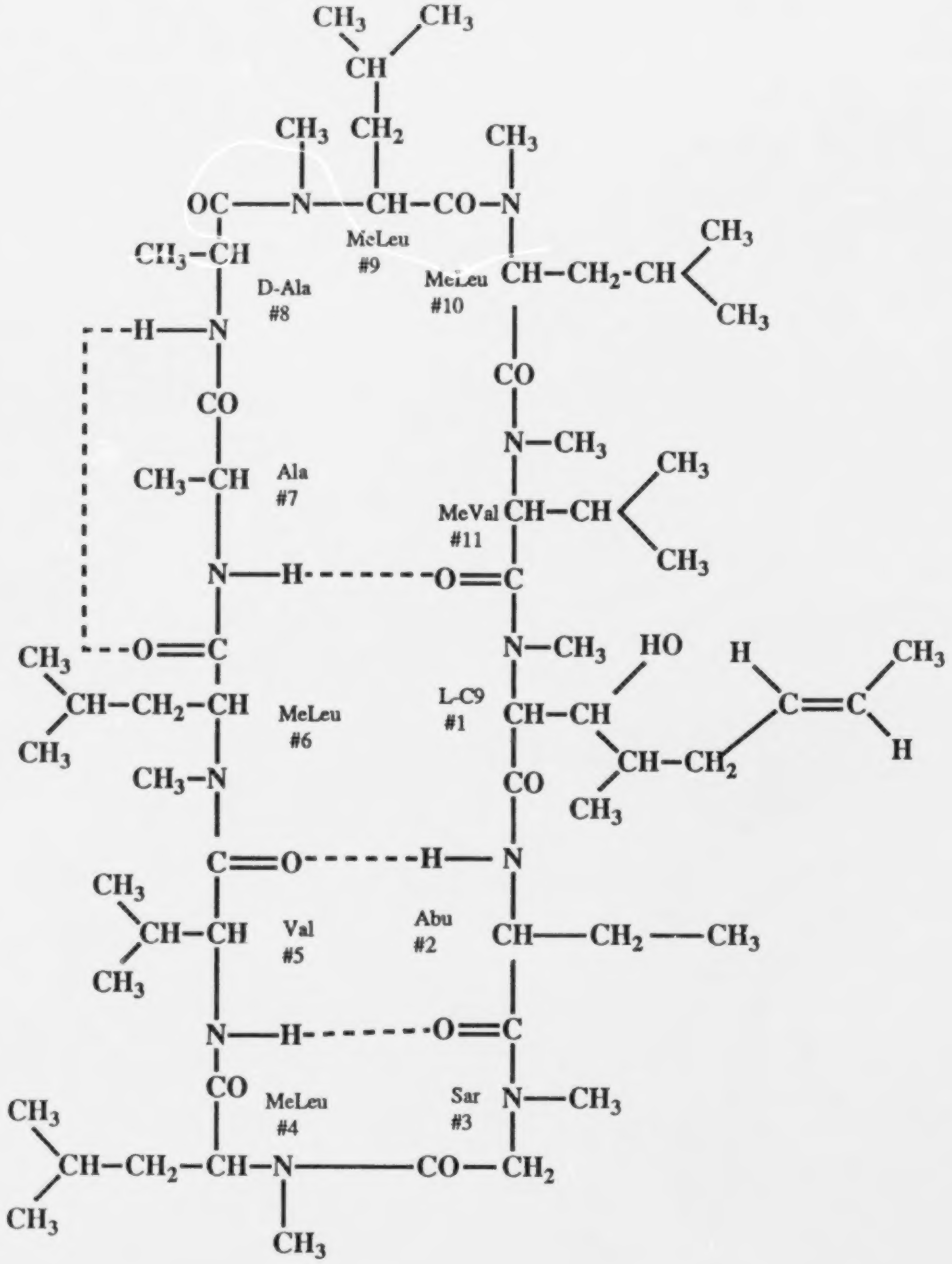
Cyclosporins are a group of secondary metabolites produced by the fungus *Tolypocladium niveum*. The production of these metabolites was initially reported by Dreyfuss *et al*. (1976) following the isolation of cyclosporins A (CyA) and C from a submerged culture of *Trichoderma polysporum*, later identified as *T. niveum*. Twenty-five naturally occuring cyclosporins designated A to Z have now been identified (Fliri and Wenger, 1990).

1.2 THE STRUCTURE OF CYCLOSPORINS

Following its discovery, the structure of CyA (Figure 1) was established by chemical degradation and X-ray crystallographic analysis of an iodo-derivative (Wenger, 1981). Cyclosporin A, like the other naturally occuring cyclosporins is a neutral hydrophobic cyclic undecapeptide consisting of L-amino acids with the exception of D-alanine in position 8. Seven of these amino acids are N-methylated. The molecule is also characterised by the presence of the unusual amino acids 2-aminobutyrate and (2S,3R,4R,6E)-2-methylamino-3-hydroxy-4-methyloct-6-enoic acid (L-C9 acid), now designated (4R)-4-((E)-2-butenyl)-4,N-dimethyl-L-threonine (MeBmt) in accordance with amino acid nomenclature (Rehácek and De-xiu, 1991). MeBmt had not been previously described and is formed by head to tail coupling of 4 acetate groups (Traber *et al*. 1989).

Cyclosporin A differs from its homologues due to variations in the amino acid sequence. These have been observed in all positions except 3 (sarcosine) and 8 (D-alanine). The most common variation is observed in position 2 in which L-2-aminobutyric acid in CyA is replaced by alanine, threonine, valine or norvaline. Other variations are due to a lower degree of N-methylation and replacement of L-C9 acid by

Figure 1: Chemical structure of cyclosporin A


D-Ala
#8
MeLeu
#9
MeLeu
#10
Ala
#7
MeVal
#11
MeLeu
#6
L-C9
#1
Val
#5
Abu
#2
MeLeu
#4
Sar
#3

3'-deoxy-L-C9, methylleucine or L-2-methylamino-octanoic acid (Fliri and Wenger, 1990).

The secondary structure of the cyclosporin molecule consists of an antiparallel ß-pleated sheet conformation of residues 1-6 which is held together by hydrogen bonding and an open loop of residues 7-11.

1.3 THE IMPORTANCE OF CYCLOSPORINS:

1.3.1 Antimicrobial and antiparasitic activity:

The initial study examining the biological activity of CyA and CyC described these compounds as weak, narrow-spectrum antifungal agents exhibiting sensitivity to only a few species of yeast and no inhibition of bacterial growth (Dreyfuss *et al.* 1976). Some strains of mucorales, ascomycetes and deuteromycetes showed differing sensitivities to the cyclosporins with *Aspergillus niger* and *Curvularia lunata* being the most sensitive. The mode of antifungal action seemed to be directed to cell wall synthesis, especially chitin synthesis. Inhibition of growth among the fungi became evident by the deformation and branching of the growing hyphal tips. The aerial mycelium and germination of conidia and spores were not affected. In sensitive yeasts, presence of cyclosporins in the growth medium led to growth rate reduction.

Cyclosporin A has been shown to inhibit the growth of *Cryptococcus neoformans*, an opportunistic pathogen found in patients with decreased T-lymphocyte mediated immune function including patients with acquired immune deficiency syndrome (Mody *et al.* 1988). Mice treated *in vivo* with immunosuppressive doses of cyclosporin A were able to survive indefinitely and also clear *C. neoformans* from their lungs more rapidly. *In vitro*, CyA directly inhibited the growth of the pathogen when administered at levels comparable to the blood levels achieved in immunocompromised mice.

Entomopathogenic activity of cyclosporins on mosquito larvae has also been reported (Weiser and Matha, 1988a). Different concentrations of cyclosporins A, B,and

C administered to larvae were found to be toxic causing mortality to more than half the population within 72 hours. CyA was the most toxic with an LC_{50} of 0.6 $\mu g\ ml^{-1}$ after 48 hours of exposure followed by CyC and CyB. It is not however known if this antiparastic nature of the cyclosporins and their inhibitory effect on *C. neoformans* are being exploited further.

1.3.2 Cyclosporin A as an immunosuppressant:

Current medical and biochemical interest in the cyclosporins is due to the strong and selective immunosuppressive nature of cyclosporin A. Registered under the trade mark Sandimmun®, CyA has been marketed since 1983 for the prevention of graft rejection in organ transplantations (Fliri and Wenger, 1990). Using CyA, the one year survival rate in heart transplant patients increased from 66% to 80% and from 65% to 91% for kidney transplants (Byrne, 1988). Cyclosporin A is also very effective in the treatment of some patients with autoimmune diseases such as endogenous uveitis, psoriasis, type 1 diabetes mellitus (insulin-dependent) and rheumatoid arthritis (von Graffenried *et al.* 1989). This is based on the premise that autoimmune diseases are T-cell driven (Talal, 1989). This function of CyA however has problems in that there is relapse of the disease after treatment is discontinued. The use of CyA in the treatment of type 1 diabetes in NOD mice and BB rats, groups that would normally develop diabetes at 3-6 months of age, totally prevented disease occurence only as long as treatment was continued.

Although the biochemical mechanism is not completely understood, it is believed that CyA suppresses immune responses by inhibiting the production of T-lymphocyte stimulating growth factors particularly Interleukin-2 (IL-2) from activated T-helper cells (Talal, 1989). Interleukin-2 is produced in response to a signal generated by processed antigens present on the macrophage surface in association with major histocompatibility complex molecules interacting with the antigen specific T-cell receptor. IL-2 interacts with specific IL-2 receptors to induce clonal T-cell proliferation. Since the balance between T-cell help and suppression, the generation of gamma-interferon and the functioning of natural killer cells are all highly dependent on IL-2 synthesis and IL-2 receptor-driven endocytosis, no cytotoxic allograft responses are mounted by the T-cells in the absence of IL-2 and therefore organ rejection does not occur.

In *Saccharomyces cerevisiae* and *Neurospora crassa*, cyclophilin, a highly conserved protein present in all eukaryotic cells has been reported as the primary cellular target for cyclosporin action (Tropschug *et al.* 1989). Mutants resistant to cyclosporin A in both organisms were found to be deficient in cyclophilin or if present, the protein could no longer bind to CyA. A similar protein, FK-binding protein (FKBP) has been identified as the binding protein for the macrolide immunosuppressant agent FK506. Rosen et al. (1990) reported that both cyclophilin and FKBP exhibit peptidyl-prolyl-*cis-trans*-isomerase (PPIase) activty. PPIase is an enzyme that catalyses the *cis-trans* isomerization of proteins and peptides, a process belived to be important in folding of proteins into their native conformations. Cyclophilin and FKBP could therefore be involved in some signalling processes that lead to T-cell activation but the molecular mechanism of this PPIase activity in lymphocyte-specific signal transduction events remains to be discovered.

It has also been suggested that CyA most likely inhibits T-cell proliferation by antagonizing a Ca^{2+} ion dependent intracellular signal transmitted from the cell membrane after interaction of the T-cell receptor with the antigen (Kay, 1989). This signal appears to be necessary for continued lymphokine production and initiation of T-cell proliferation. One step in T-lymphocyte activation in which calcium is essential is the induction of the synthesis of the messenger-RNA for IL-2 and other lymphokines. The mechanism by which CyA acts to inhibit the Ca^{2+} ion signal is however still unclear although in specific situations, CyA has been shown both to enhance and to inhibit the transport of

Ca^{2+}across membranes in cells other than lymphocytes including antigen presenting cells (Kay, 1989).

1.3.3. Structure-activity relationship:

For high immunosuppressive activity, the intact structure of MeBmt is essential. Replacement of this amino acid by N-methyl-L-leucine (CyO) or N-methyl-2-aminooctanoic acid (CyZ), for example, reduces the activity drammatically. Activity is also reduced in the absence the hydroxyl group as well as the olefinic double bond. MeBmt is not, however, solely responsible for the immunosuppressive action. Loss of activity is observed when 2-aminobutyric acid in position 2 is replaced by amino acids like L-alanine (CyB) and valine (CyD). Changes in conformation and structure of the cyclosporin loop are the other factors that affect the activity as observed by the introduction of an additional hydrogen bond between amino acids 8 and 11, replacement of L-alanine in position 7 by D-alanine and reduction of the loop by the removal of D-alanine. From these studies it is clear that cyclosporin activity is associated with a large part of the molecule that probably includes amino acids 1, 2, 3, 9, 10 and 11 and, variations are permitted only in a few positions without significant loss of activity.

1.4 BIOSYNTHESIS OF CYCLOSPORINS:

Cyclosporins belong to a group of amino acid derived secondary metabolites known as peptide antibiotics or recently, bioactive peptides (Kleinkauf and von Döhren,1987). Bioactive peptides occur as groups of closely related compounds, the members of which show only minor variations in the nature and sequence of constituent amino acids. They differ from proteins and peptide hormones in that they contain unusual amino acids. Their cyclic structures do not involve sulfur bridges, and, in addition to L-amino acids, contain D-amino acids. A further peculiar property is the occurence of N-methylated peptide bonds in such compounds. Other than cyclosporins, these metabolites include Gramicidin S, linear gramicidins, alamethicin, enniatin, tyrocidins and bacitracins and are all synthesized by the non-ribosomal mechanism.

RNA-free peptide formation was initially demonstrated in 1960 in the synthesis of the cyclic decapeptides Gramicidin S and tyrocidin (Kleinkauf and von Döhren, 1981). This reaction, which is catalyzed by multifunctional enzymes, can be distinguished from the ribosomal process by its ability to resist the action of RNAase and protein synthesis inhibitors like chloramphenicol and streptomycin (Vater, 1990). Two pathways have been characterized for the non-ribosomal biosynthesis of bioactive peptides (Vater, 1990). The first pathway is one in which the multienzymes activate their substrate amino acids in two steps. This involves activation of constituent amino acids as aminoacyl adenylates and the subsequent transfer of the aminoacyl to reactive sulfhydryl groups at the specific thiotemplates to form thiolesters. It has been postulated (Kleinkauf *et al* . 1971) that the substrates which are continuously provided at the reaction centres are linked together in a series of transpeptidation and transthiolation processes by interaction of an internal central phosphopantetheine carrier with the peripheral thiol groups at the reaction centres. This has been called multienzyme thiotemplate mechanism since all activated intermediates formed are thiolesters.

The second non-ribosomal mechanism involves a one-step phosphate activation mechanism. In this case, elongation proceeds by the addition of nonactivated acceptor amino acids to carboxyl activated intermediate peptides attached noncovalently to the reaction centres as aminoacyl phosphates. An example of a peptide synthesized in this manner is mycobacillin, a tridecapeptide from *Bacillus subtilis* B3

The presence of N-methylated bonds and unusual amino acids 2-aminobutyric acid, D-alanine and the L-C9 acid was an initial indication that cyclosporins might be synthesized by the non-ribosomal mechanism. This had been established in other fungal peptides like alamethicin and enniatin (Zocher *et al.* 1984). Although initial attempts to synthesize cyclosporins using cell free extracts were unsuccessful, it has now been established that CyA and its homologues are synthesized by a single multifunctional enzyme from their precursor amino acids using the thiotemplate mechanism (Lawen and Zocher, 1990). From this and other information available on the synthesis of other peptides it is likely that cyclosporin synthesis proceeds as discussed below and shown in Figure 2.

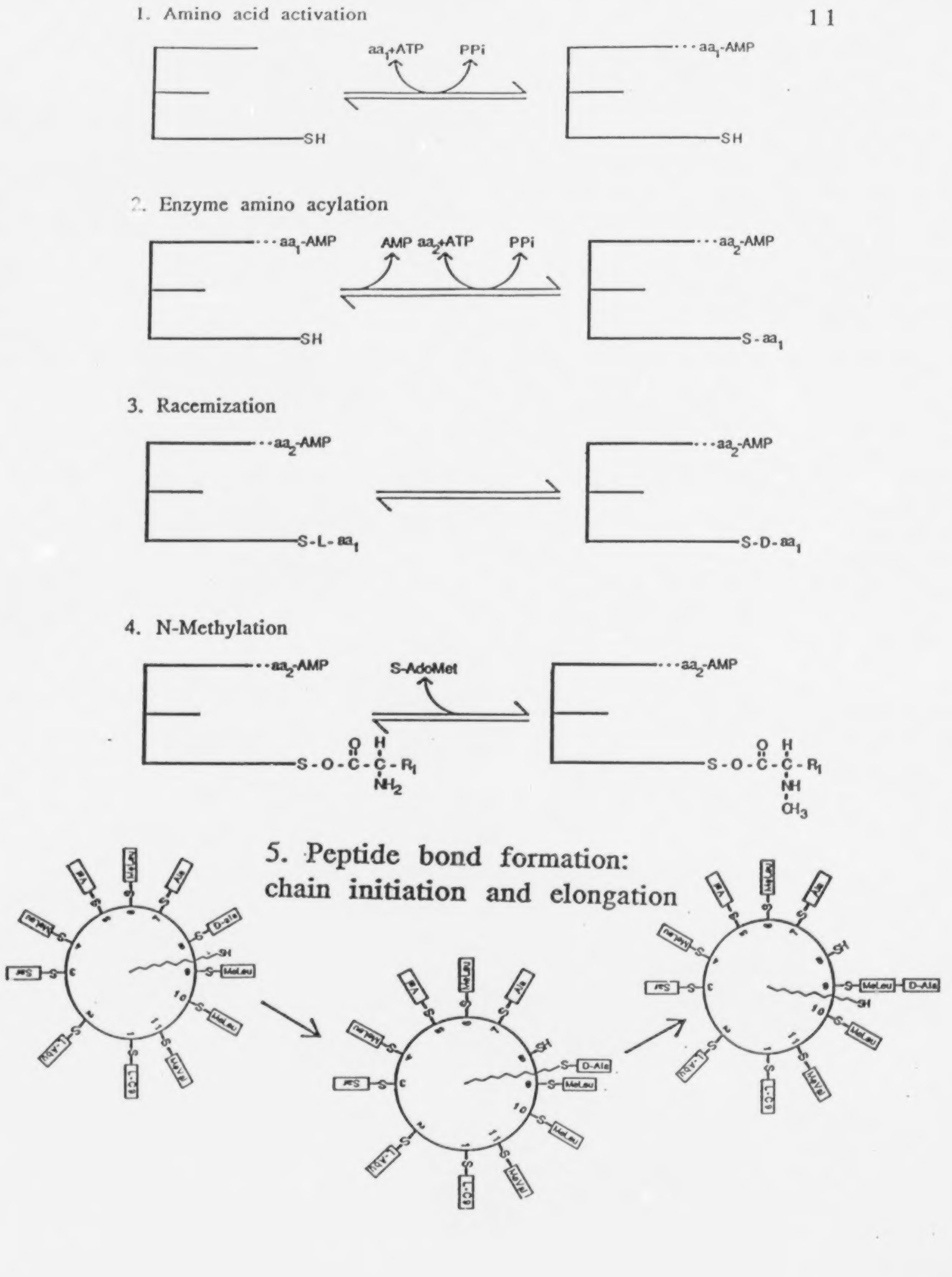
AMINO ACID ACTIVATION: This step involves the activation of individual amino acids by ATP in the presence of Mg^{2+} to form amino acyl adenylates which are bound non-covalently to the enzyme. The reaction proceeds by cleavage of alpha-beta phosphate bonds of ATP. An analogous step also occurs in the ribosomal mechanism.

AMINO ACYLATION OF ENZYME: This is the first unique reaction of the non-ribosomal biosynthetic mechanism. In this reaction, the amino-acyl moiety of the adenylate is transfered and covalently linked to the specific thiol group on the enzyme.

RACEMIZATION OF AMINO ACIDS: In cases where the amino acid is present in the molecule in the D-configuration, racemization from the L form occurs at this stage. The configuration of the D-alanine in cyclosporin at the time of amino acid activation has not been clearly defined although both D-alanine and D-serine have been shown to be substrates for cyclosporin synthetase (Traber *et al.* 1988; Lawen and Zocher, 1990). Enzyme studies with other synthetases have shown that some sites with missing stereoselectivity accept both the L and D isomers but utilize only the D form in further reactions. Most systems select the L-form and should therefore possess an epimerase function.

N-METHYLATION OF PEPTIDE BONDS: Methylated peptide bonds are mostly found in cyclic peptides of Actinomycetes and some Hyphomycetes. They are thought to be important in the the stabilization of peptide bonds against proteolytic cleavage and may influence conformational stabilities (Kleinkauf and von Döhren, 1987; Billich and Zocher, 1990). In cyclosporin biosynthesis, N-methylation occurs at the level of thioesterified amino acids. The methyl groups originate from methionine with S-

Figure 2: Model for possible mechanism of cyclosporin biosynthesis



1. Amino acid activation

2. Enzyme amino acylation

3. Racemization

4. N-Methylation

5. Peptide bond formation: chain initiation and elongation

aa1+ATP
PPi
SH
···aa1-AMP
AMP
aa2+ATP
···aa2-AMP
S-aa1
S-L-aa1
S-D-aa1
S-AdoMet
S-O-C-C-R1
O
H
NH2
NH
CH3
S
SH
MeLeu
Ala
D-Ala
MeVal
Sar
10
11

adenosyl-L-methionine serving as the methylating coenzyme. The methyltransferase activity is an integral entity of the enzyme.

PEPTIDE BOND FORMATION: Peptide bonds are formed by initiation, elongation and termination reactions. Elongation occurs by head growth in which the activated carboxyl group of a donor amino acid or intermediate peptide reacts with the amino group of the enzyme bound acceptor amino acid. The central role in elongation reactions is attributed to 4'-phosphopantetheine, a cofactor functioning as a swinging arm in which it alternates as an acceptor of the growing peptide chain and donor of the peptide to the next thioester-linked amino acid sequence. In cyclosporin synthetase, the cofactor is covalently attatched to the multienzyme. 4'-phosphopantetheine also serves a transport function in many systems involving carboxylated groups.

1.5 PRODUCTION OF CYCLOSPORINS:

1.5.1 Organisms producing cyclosporins:

Since the first description of the cyclosporins and their production by *Tolypocladium niveum*, there have been very few reports of their production by other organisms. *Tolypocladium cylindrosporum*, *Beauvaria bassiana*, *Fusarium solani*, and *Neocosmospora vasinfecta* have been reported to produce cyclosporins (Aarnio and Agathos, 1989) but no further work has been published on production in these systems. To date, all the work involving cyclosporin production has been carried out using *T. niveum*, also classified as *Tolypocladium inflatum* (Gams, 1971) and *Beauvaria nivea* (von Arx,1986). The name *T. niveum* will be used throughout this work.

Tolypocladium niveum is a fungal strain belonging to the class hyphomycetes (Bissett, 1983). It is characterized by slow-growing, white, floccose colonies and spherically swollen phialides. The phialides are borne on hyaline, short branched conidiophores and occur singly or in verticils of two to five. They terminate as narrow necks bearing single-celled subglobose conidia. The conidia measure an average of 2.3 X 1.7 µm and accumulate in small heads at the tips of the phialides.

1.5.2 Directed synthesis of cyclosporin:

Cyclosporins are produced as intracellular products in submerged cultures of *T. niveum.* Their fermentation conditions, isolation and characterization were initially outlined by Dreyfuss et al. (1976). Thereafter, it was shown that cyclosporin synthesis could be directed towards a specific form by incorporating a particular amino acid into the growth medium (Kobel and Traber, 1982). Exogenous addition of the amino acids DL-2-aminobutyric acid, L-alanine, L-threonine, L-valine and L-norvaline, each of which can be incorporated in position 2 of the molecule led to directed biosynthesis of cyclosporins A, B, C, D and G respectively. Although an increase in total Cy production was observed in all cases except with L-alanine, only DL-2-aminobutyric acid showed total suppression of all the other cyclosporins in favour of CyA. The incorporation of amino acids not normally found in the cyclosporins has also been reported (Traber *et al.* 1989). Feeding experiments using L-ß-cyclohexylalanine, DL-allylglycine and D-serine, led to the replacement of the L-C9 acid in position 1, L-2-aminobutyric acid in position 2 and D-alanine in position 8 respectively.

Other studies on directed biosynthesis of cyclosporin showed that addition of exogenous L-valine to both synthetic and semisynthetic medium led to enhanced CyA and total cyclosporin production (Lee and Agathos, 1989). In these experiments, L-valine did not support the synthesis of cyclosporins C and D and did not preferentially direct synthesis to the formation of CyD in contrast to the results of Kobel and Traber. An increase in CyA was also observed in synthetic medium supplemented with L-leucine and glycine but was suppressed by L-leucine in semisynthetic medium containing peptone. Glycine had no effect in this medium. Sarcosine and L-methionine reduced CyA production by 50% and 70%, respectively. A more dramatic decrease was observed by the addition of L-valine and L-methionine together suggesting that one or more methylation steps by L-methionine mediated through S-adenosyl-L-methionine might control the synthesis of CyA through a feedback mode. Further studies to determine the role of L-valine in CyA synthesis showed that in supplemented medium, CyA synthesis continued even after the endogenous L-valine levels, which were thought to activate production, were depleted (Lee and Agathos, 1991). This led to the suggestion that an intermediate compound accumulated from the high L-valine pool during exponential growth may be utilized for production of the metabolite after depletion of endogenous valine. Increases in the internal pool of a supplemented amino acid have been observed with tryptophan and methionine in the production of ergot alkaloids by *Claviceps purpurea* and cephalosporin by *Cephalosporium acremonium* respectively (Matsumura *et.al.*. 1981, Krupinski *et al*. 1976). In line with the cited literature, this study led to the conclusion that the apparent stimulation of CyA formation by exogenous addition of L-valine and the prolongation of the production phase beyond the period of active growth, suggest that L- valine may have an inducing role for Cy A synthetase. An increase in total cyclosporin was also observed in cultures of five different strains of *T. niveum* supplemented with L-valine, L-alanine and L-norvaline (Isaac *et al.* 1990). Directed synthesis of CyG by DL-norvaline was observed but there was no exclusive formation of CyA by DL-aminobutyric acid.

These studies have shown that precursor directed biosynthesis is not only a useful and efficient way of improving the cyclosporin yield but also a tool for preparing cyclosporin analogues not encountered in nature. The successful incorporation of constituent and foreign amino acids, however, demonstrates the low specificity of the cyclosporin synthetase, a characteristic shared by other multienzymes using the non-ribosomal pathway.

1.5.3. Nutritional strategies and batch kinetics:

Another aspect of cyclosporin production that has been reported involves the optimization of cyclosporin formation by varying the nutritional and physical conditions during fermentation. These studies have shown that cyclosporin production can be supported by a broad spectrum of carbon compounds including pentoses, hexoses, disaccharides, polysaccharides and organic acids (Agathos *et al.* 1986; Isaac *et al.* 1990). Analysis of a variety of carbon sources in both studies showed that sorbose supported the highest cyclosporin production. It was also noted that carbon sources highly favoured for biomass production by *T. niveum* did not generally provide the physiological state necessary for optimal secondary metabolite production and that increasing the concentration of the carbon source did not necessarily increase the productivity (Agathos *et al.* 1986).

An investigation of the nitrogen sources revealed that inorganic nitrogen sources such as ammonium and nitrate salts did not support cyclosporin production (Agathos et al. 1986). High metabolite formation was obtained using Bacto-peptone and vitamin assay casamino acids at a 1% concentration (Agathos *et al.* 1986: Isaac *et al.* 1990) an indication that organic nitrogen sources are more favoured for cyclosporin production than inorganic ones.

The batch kinetics of cyclosporin fermentation were also examined. Agathos *et al.* (1986) showed that there was no significant separation of trophophase and idiophase as cyclosporin could be detected almost from the beginning of the fermentation. In agreement with Dreyfuss *et al.* (1976), an initial rapid growth phase associated with rapid uptake of the carbon source was observed. This was followed by a slower growth rate during which most of the Cy production occurred. The production of Cy was found to be essentially complete after ten days, beyond which there was extensive hyphal fragmentation and lysis of mycelia. Growing the cultures at 15°C instead of 27°C resulted in increased Cy production in some cases (Agathos *et al.* 1986) but not in others (Isaac *et al.* 1990).

1.5.4. Cell free synthesis:

The description of cell-free synthesis as an alternative for Cy production was not reported until 1986 when Zocher *et al* reported the isolation of an enzyme fraction with an apparent molecular weight of 700 KD. This enzyme was capable of catalyzing the activation of the amino acids present in the CyA molecule as thioesters via the corresponding adenylates, an indication that the isolated enzyme may be involved in the non-ribosomal biosynthesis of cyclosporin. In addition, the enzyme also catalyzed the N-methylation of thioesterified L-valine, L-alanine and glycine residues using S-adenosyl-L-methionine. For unclear reasons however, cyclosporin A synthesis was not observed. Instead, the diketopiperazine cyclo-(D-alanine-Meleu) from D-alanine and Meleucine, both of which are components of the cyclosporin molecule was formed. Recently, Dittmann *et al.* (1990) have reported the production of this compound by a mutant strain incapable of producing cyclosporin.

In 1987, isolation of an enzyme capable of synthesizing CyA *in vitro* was reported using a different strain of *T.niveum* (Billich and Zocher,1987). In addition, in vitro synthesis of cyclosporins B, C, D, and G was achieved by replacing L-2-aminobutyric acid with the respective position 2 amino acids. Further work by the same group revealed that a variety of cyclosporins including those not encountered in nature could be synthesized *in vitro* using cell-free extracts (Lawen *et al.*1989). Recently, it has been confirmed that cyclosporin synthesis follows the thiotemplate mechanism (Lawen and Zocher,1990). Further purification and characterization of cyclosporin synthetase has shown that the enzyme occurs as a single non-glycosylated band of about 650 kDa and is solely responsible for synthesis of CyA and its homologues. This is in contrast to the results of Fode(1990) in which four proteins capable of activating the constituent amino acids were reported. The presence of 4'-phosphopantetheine which occurs as a prosthetic group on the synthetase was confirmed in both studies.

1.6. STRAIN IMPROVEMENT PROGRAMME:

Since the initial work on penicillin production in *Penicillium chrysogenum,* the use of physical and chemical agents to obtain high metabolite producing mutants has formed the backbone of strain development programs. The success of this process has been demonstrated by the increase in penicillin yields from 100 units ml^{-1} in 1943 to 50 000 units ml^{-1}in 1979 (Queener and Swartz, 1979). Strain improvement is necessary not only for increasing titre production but can also lead to establishment of strains which produce new or modified products. The process also plays a crucial role in the understanding of the biosynthetic pathways and control mechanisms that govern those pathways. Mutagens act primarily by inducing lesions or modifying the base sequence in the DNA molecule. Failure to repair these alterations or errors introduced by the repair mechanisms results in a mutation.

1.6.1. Ultra-violet (UV) mutagenesis:

UV light induces mutagenesis by the creation of covalent linkages between adjacent pyrimidine residues, on the same DNA strand to form dimers (Peberdy, 1988). The dimers are formed through covalent linkage of the 5,6-carbon atoms of adjacent thymines or cytosines to form a cyclobutane ring (Hanawalt, 1977). Pyrimidine dimer presence in template DNA strands blocks DNA replication and unless it is repaired, it can be lethal. Although the DNA repair mechanisms in *Tolypocladium* have not been studied, it is likely that these are similar to those reported for bacteria as well as yeast. In general, the DNA repair process following UV-induced damage involves the error-free and error-prone mechanisms. In *Eschericia coli,* the error-free mechanism is constitutive and the error-prone is inducible. Three error-free repair systems have been identified i.e. photoreactivation, excision repair and recombination repair.

The photoreactivation system consists of an enzyme which is capable of recognizing pyrimidine dimers on the DNA strand. In the presence of a dimer, the enzyme will bind to the dimer-containing region and photocatalytically split it. The enzyme is then released leaving an undamaged DNA molecule which can replicate normally. In some organisms, the system may be responsible for correctly repairing up to 60% of the UV induced lesions (Hopwood, 1970).

Excision repair involves the nicking of the DNA strand containing the dimer, on the 5' side of the dimer. The dimer is then removed together with the adjacent nucleotides using the 5' to 3' exonuclease activity of DNA polymerase 1. The resulting gap is filled by the polymerase using base pairing specificity from the complementary strand and final joining is accomplished by the polynucleotide ligase (Hanawalt, 1977).

The recombination repair mechanism operates in cases where a dimer is not repaired prior to replication (Moat and Foster, 1988). DNA polymerase 1 does not recognize the dimer as a template and will therefore not replicate the DNA in the region of the dimer. This results in a gap in the new strand in the region opposite the dimer. The gap is repaired by recombining the appropriate region from the intact sister chain after which the dimer is removed by excision repair.

In cases where the error-free mechanism fails to operate, the error-prone mechanism, also called the SOS pathway, becomes functional. This mechanism is responsible for all or most mutations occuring after UV damage (Radmann, 1977). In *E. coli* the SOS pathway is a recA and lexA dependent process which is induced by the interruption of DNA synthesis due to unrepaired DNA damage. The SOS repair system acts by overriding the normal editing function of the DNA polymerase to allow replication where the cell would otherwise die. This results in incorrect copying of the DNA base sequence thus resulting in a mutation.

1.6.2 Mutagenesis using epichlorohydrin:

Epichlorohydrin is a chemical mutagen that has been found to be mutagenic due to alkylation of C-6 amino group of adenine, N-1 and C-2 amino group of guanine and N-3 of cytosine in the DNA molecule. In general, alkylating agents decompose when introduced in the cell producing alkyl-diazonium ions which in turn react with nucleophilic sites in DNA to produce a variety of N-alkylated and O-alkylated nucleotides (Schendel *et al.* 1983). This direct action on the DNA molecule causes changes in one base or another thus resulting in faulty pairing or other changes which unless repaired can lead to mutations.

The adaptive-response is the main mechanism involved in repairing damage caused by alkylation of the DNA molecule. The mechanism consists of two enzyme systems, alkyltransferases and N-glycosylases, which carry out repairs by direct transfer of the modifying alkyl group from DNA to the repair protein or by a direct cleavage of the glycosidic bond of the alkylated base by a specific glycosylase (Helland *et al.* 1987). The cleaved glycosidic bonds can then be removed by apurinic or apyrimidinic-specific (AP-specific) endonucleases which cleave either at the 3' or 5' side of the AP site. Repair then proceeds through the excision-repair mechanism. Adaptive-response is an inducible mechanism and appears to be error-free but has a drawback in that it has a limited capacity and ceases to function when too much alkylation has occurred thus increasing the chances for mutation. Its main target is O^6-methylguanine, a miscoding lesion and to a less extent N-alkylated purines. Unlike in UV-induced mutagenesis, the specific details on how the repair of epichlorohydrin alkylated bases eventually leads to mutations remain unclear.

The mismatch repair mechanism is another way by which damage caused by exposure to alkylating agents is repaired. This mechanism is a postreplicative DNA repair system that recognizes mismatched bases that have eluded the 3' to 5' proofreading function of the DNA polymerase. During DNA replication, O^6-methylguanine present on the template strand is interpreted as adenine thus causing a GC to AT transition on the daughter strand. In order to correct this sort of mismatch, some form of discrimination is needed for the repair system to recognize newly synthesized strands from the parental strands. This is accomplished by the DNA adenine methylase (*dam*)- instructed pathway which methylates the N6-adenine of DNA at GATC sequences. Through this process, the newly synthesized strands which are undermethylated relative the parental strands can be distinguished and excised. The process occurs prior to a subsequent round of replication in order to prevent a mutation.

1.6.3. Screening and selection of mutants:

Following random mutagenesis, a number of mutants are produced and in order to generate mutants with the desired phenotype, rational methods of selection are applied. These take advantage of the knowledge of the biochemistry of the product to be formed. For most secondary metabolites however, this information is not always available and therefore indirect selection has to be applied. In bioactive peptide synthesis, one of the approaches that has been used is to select the mutants for resistance against structural amino acid analogues.

The production of primary metabolites including those involved in secondary metabolite formation is affected by the existence of feedback regulatory mechanisms by enzymes early in the synthetic pathway. Limiting the amount of precursor will interfere with secondary metabolite formation. This is based on the premise that, in most cases, the amount of secondary metabolite produced depends on the excessive production of suitable precursors by corresponding pathways of primary metabolism (Zahner and Kurth, 1982). By selecting for resistance against antimetabolites, improvements in secondary metabolite titres have been achieved due to overproduction of the required natural metabolites (Rolands,1983). This overproduction results from the fact that in the presence of an antimetabolite, the formation of the genuine product by the organism is prevented due to the inhibition or repression of the enzyme(s) by the antimetabolite. The antimetabolite is, however, unable to substitute for the genuine product in its essential role within the cell. In order to survive, the cell acts by overcoming the regulatory mechanisms thus leading to uncontrolled production of the genuine product.

1.6.4. Recombination:

Other methods by which strain improvement can be achieved include parasexual techniques. These techniques represent a series of events by which genetic elements contained in two separate genomes are brought together in one unit. In industrial organisms, two parasexual techniques i.e. parasexual cycle and protoplast fusion are used to transfer information between asexual fungi. The parasexual cycle is a technique used to transfer genetic elements between hyphae of fungi grown together under laboratory conditions while protoplast fusion involves exchange of genetic information between organisms by fusion of their protoplasts. Both methods can be applied to strains independent of or following mutagenesis especially when the latter can no longer generate higher levels of the desired metabolite. Recently, a more direct method of manipulation of the fungal genome using *in vitro* recombination of DNA molecules has been developed. This technique is an important research tool for understanding the genetic structure of an organism. It is also of major importance in the construction of new organisms for practical applications. One of the foundations of recombinant genetics is a well defined and described genetic map of the organism. This is important in that it can aid in the identification and isolation of genes of interest. Also, knowledge of chromosomal location for key biosynthetic genes in industrial strains may be useful in rational strain improvements employing integrative transformation systems. For most fungi, including *T. niveum,* genetic maps are not available.

One way by which the physical maps of gene locations can be obtained is through the isolation of chromosomes using pulse-field gel electrophotesis (PFGE). Gene locations can then be identified through hybridization analyses of the chromosomes.

1.7. RESEARCH OBJECTIVES:

To date most of the work on cyclosporin production has been carried out using *T. niveum* NRRL 8044 or its mutants. The strain is also deposted as ATCC 34921. Except for the work done in our laboratory, little information is available on cyclosporin production by other strains of *T. niveum*. Cyclosporin production studies in our laboratory were initiated after the failure to obtain reproducible levels of metabolite production using strain NRRL 8044 (Isaac *et al.* 1990). In this study, production of cyclosporin by various wild type strains and species were compared under different conditions and reproducible methods for obtaining cyclosporin were sought. This was followed by another study aimed at the identification and purification of cyclosporin producing enzymes and isolation of the genes coding for these enzymes.

As part of a continued programme aimed at improving cyclosporin production and understanding the physiology of its production among the various strains of *T. niveum* this project was set out with four objectives.

The first objective was to isolate mutants of *T. niveum* producing higher levels of cyclosporin using UV and epichlorohydrin induced mutagenesis. The mutants were directly selected using 4-aza-DL-leucine, an analog of the amino acid L-leucine.

The second stage of the project was aimed at investigating how growing the mutants on various carbon sources at different concentrations affects cyclosporin production. The growth of the organisms and how this affects cyclosporin production with time was also investigated and morphological characteristics that could be responsible for increased Cy levels were sought. Another aspect of this part was to determine the effect of supplementing the production medium with constituent and non-constituent amino acids. The third stage of the project was involved in examining the enzyme(s) of cyclosporin production. Although this area had been studied to some detail by Fode (1990) and Lawen and Zocher (1990), it is still unclear as to whether cyclosporin synthatase(s) occur as a single molecule identified as a single band on sodium-dodecyl sulphate-polyacrylamide gels or consists of greater than one molecule. It is also not possible to deduce from the available literature whether the variation in the number of bands could be due to strain variability.

The last part of the project was aimed at separating chromosomes of *T. niveum* using pulse field gel electrophoresis. This would serve as a basis for examining the structural and functional organization of chromosomal DNA of *T. niveum* .

2. MATERIALS AND METHODS:

2.1 Strains:

The strain used in most of this study was *Tolypocladium niveum* UAMH 4002 or mutants thereof. This strain was obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH) and was originally isolated from muskeg soil obtained from Tuktoyaktuk in the Northwestern Territories. Strain UV-98 is a UV mutant of UAMH 4002 obtained after a single round of mutagenesis and EPI-74 is an epichlorohydrin mutant of UV-98. For a few experiments, strain B-5, a UV mutant of *T. niveum* UAMH 2472 was used (Fode, 1990).

2.2 Maintenance of cultures:

The cultures were maintaned as spore suspensions in 20% (w/v) sterile glycerol at -75°C. The spores were obtained from cultures grown on malt extract (2%)-yeast extract (0.4%) agar (malt-yeast agar) plates and incubated at 27°C for 10 days. Spores were collected by gently scraping the surface of the colony with a speader using 4 mL of glycerol per plate. The combined suspension was then dispensed in 1 mL vials and stored. The spore concentration was determined by the viable spore count method using serial dilutions of the combined suspension.

2.3 Fermentation conditions:

Cultures for routine cyclosporin production, enzyme production and protoplast isolation were grown in 500 mL Erlenmeyer flasks containing 100 mL of medium and incubated at 27°C on a rotary shaker at 200 rpm. The medium consisted of 2% carbon source, 1% Vitamin Assay Casamino Acids, 1% potassium orthophosphate monobasic and 1% potassium chloride in distilled water, adjusted to pH of 5.2 before autoclaving for 15 minutes. Growth was initiated by inoculating preculture medium with 10^8 spores.

After 72 hours of growth, the preculture was homogenized for 30 seconds in a Sorvall Omnimixer and 10 mL (10% v/v) of the homogenate used as inoculum for production cultures (Isaac *et al.* 1990). Incubation periods varied depending on the experiment.

2.4 Extraction and analysis of cyclosporin:

After the intended period of growth, the volume of each culture was reconstituted to 110mL with distilled water and homogenized for 30 seconds. Extraction of cyclosporin was carried out by adding 10 mL of the sample suspension with an equal volume of ethyl acetate. Separation and quantitation were carried out by high-pressure liquid chromatograph (HPLC) using a reverse-phase column (Isaac *et al.* 1990). The only exceptions were that temperature of the column was maintained at 70°C and sample injection was carried out using the Waters Intelligence Sample Processor (WISP). All data are reported as the sum total of cyclosporins A and C which were formed in the ratio 7:3 in unsupplemented media.

2.5 pH and biomass determination

Measurements of pH were carried out using a Fisher Accumet model 230 pH/Ion meter. Mycelial biomass was determined by filtering 5 mL of culture through Millipore HA filters (0.45µm). The filters were dried at 70°C for 16 hours and weighed.

2.6 Mutation and selection:

2.6.1. UV-mutagenesis:

Spores of *T. niveum* UAMH 4002 were washed twice with sterile distilled water to remove the glycerol and resuspended in 10 mL water. The suspension, containing approximately 10^7 spores/mL was placed in a sterile glass petri dish and irradiated with a UV lamp (254 nm and 700µWatt/cm^2 intensity) from a distance of 15 cm for 10, 40, 120 and 300 seconds. Samples collected at these times were each diluted serially and 0.2 mL aliquots plated on malt-yeast agar in triplicate, along with a control sample removed before UV exposure. The samples were incubated for 72 hours at 27°C after which the kill rate was determined.

2.6.2 Epichlorohydrin-induced mutagenesis:

As in UV- induced mutagenesis, the initial approach to this experiment was to establish the kill rate of the organism. Spores of strain UV-98 were washed twice with sterile distilled water to remove the glycerol and resuspended in 9 mL of water. 1 mL volumes of various epichlorohydrin concentrations were then added directly to the 9ml spore suspensions. The suspensions, each containing approximately 10^8 spores/mL were incubated with gentle agitation at 27°C. The treatment of spores was stopped by removing 1 mL of the suspension at various time periods. Epichlorohydrin was removed from the samples through centrifugation and washing the cells twice with distilled water. The cells were then resuspended in 1 mL water, serially diluted and plated on malt-yeast agar. A control sample removed before addition of the mutagen was similarly treated. Colonies germinating after 7 days of incubation at 27°C were used to determine the kill rate.

2.6.3 Determination of 4-aza-DL-leucine concentration:

The concentration of 4-aza-DL-leucine used for mutant isolation was determined by plating the organism on medium containing various concentrations of the analogue. Washed spores of UAMH 4002 or its mutant were serially diluted and plated on minimal media containing 1% fructose, 0.3% $NaNO_3$, 0.5% $MgSO_4$, 0.05% KCl, 0.1% K_2HPO_4, 0.001% $FeSO_4$ and 1.5% agar. The plates were incubated at 27°C for 72 hours after which the inhibitory levels were determined.

2.6.4 Isolation and selection of mutants:

Mutants were isolated by exposing the spores to the mutagen at 99% kill rate level, as determined above, and plating the spores on minimal medium containing an inhibitory concentration of 4-aza-DL-leucine. Colonies germinating after 7 days were picked using toothpicks and inoculated onto master plates containing malt-yeast agar. The plates were incubated for 3 days after which each colony was then scraped off and inoculated into 100 mL of the cyclosporin production medium. The cultures were incubated for 15 days before harvesting for cyclosporin analysis. The incubation and cyclosporin analysis conditions were as described in 2.4 and 2.5 above.

2.6.5 Strain stability:

The stability of mutants was determined by checking for variability in cyclosporin production on a monthly basis using spores stored at 5°C, -20°C and -75°C.

2.7 Purification and analysis of cyclosporin synthetase(s):

2.7.1 Preparation of cell free extracts:

Cell free extracts were prepared by filtering contents of 40 flasks grown in production media for 48 hours. The mycelia, collected through Schleicher and Schüll Inc. 520B filters were then washed with 4L of 50mM Tris-HCl, pH 7.5 and 0.1M KCl, frozen in liquid nitrogen and lyophilized overnight. The dried cells, averaging 12g were then ground with sand to a fine powder and the enzyme extracted by one of the two methods.

In the first method, the ground cells were mixed for 15 minutes or more with 100 mL extraction buffer consisting of 100mM 3-(N-Morpholino)propanesulfonic acid (MOPS)-KOH pH 7.5, 50mM KCl, 30mM 2-mercaptoethanol, 1mM EDTA, 5mM $MgCl_2$, 1mM phenylmethylsulfonylfluoride(PMSF) and 50% glycerol. After a homogeneous mixture was obtained, the suspension was centrifuged for 20 minutes at 27,200 x g to obtain the cell free extract. The extract was then treated with polyethyleneimine(PEI) to a final concentration of 0.05% so as to precipitate nucleic acids. The solution was allowed to stir for 15 minutes after which it was centrifuged as before but for 15 minutes. The resultant solution was then diluted with dilution buffer containing 100mM MOPS-KOH, pH 7.5, 50mM KCl and 1mM dithioerythreitol (DTE) to reduce the glycerol concentration to 20%.

The proteins in the extract were obtained by precipitation with ammonium sulfate and collecting the material precipitating in the 0-20%, 20-35% and 35-50% fractions. Each of the protein samples was then resuspended in 3 mL of dilution buffer supplemented with 20% glycerol and desalted on 10 mL Bio-Rad Econo-Pac desalting column. The 4 mL eluents were at this stage subjected to SDS-PAGE for enzyme detection or assayed for enzyme activity. Occasionally the extracts were subjected to gel filtration chromatography to further purify the proteins.

The second method of extraction was carried out as described by Lawen and Zocher (1990) but using the same volumes of buffer and centrifugation conditions described in the preceding paragraph. Also other than using different buffers, the proteins were precipitated and desalted as descibed above.

2.7.2 Gel filtration chromatography:

Gel filtration was carried out using Sephacryl®S-200 (Pharmacia) packed in a column to a volume of 145 mL and equilibrated with elution buffer. The column was run at a flow rate of 1.2 mL/min. using an LKB Microperpex peristaltic pump. Desalted samples were applied to the column and 3 mL fractions collected on the LKB model model 2111 Multirac fraction collector. The A_{280} of each fraction eluted after the void volume was then taken to determine which fractions contained proteins.

2.7.3 SDS-PAGE:

Fractions containing proteins were pooled and concentrated by ultrafiltration using an Amicon with YM-100 membrane and their protein concentration determined using the dye-binding method (Bradford,1976). The samples were then subjected to Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) following the Laemmli system (Blackshear,1984). Samples were prepared by mixing the enzyme fraction with 0.1% bromophenol blue, 40% sucrose, 20% SDS and 35% 2-mercaptoethanol in the ratio 4:2:2:1:1 respectively. The final protein concentration in the sample was 100 µg/mL and SDS-PAGE was carried out on 3.5% to 6% or 2 to 15% gradient gels. The molecular weight of the separated proteins was determined with the use of markers containing ovalbumin (43,000), bovine serum albumin (67,000), phosphorylase b (97,400), ß-galactosidase (116,250) and myosin (205,000). The proteins were identified by the silver stain procedure as described by Merril *et al.* (1984) or by staining with 0.1% Coomassie Blue in 50% methanol and 10% acetic acid.

2.7.4. Determination of cyclosporin synthetase activity:

The activity of the enzyme was determined by the ^{14}C amino acid binding assay or by the *in vitro* synthesis of cyclosporin. The thioester formation reaction was carried out by incubating 100 µL of enzyme fraction in a total volume of 160 µL with 15 mM $MgCl_2$, 15 mM ATP, 0.1M Tris-HCl pH 7.5, 1 mM DTE and 2 µM ^{14}C-L-leucine. The reaction mixture was then incubated at 27°C for a total of 30 minutes during which samples were collected every 5 minutes and the reaction stopped by adding 2 mL of 7% trichloroacetic acid. After 30 minutes on ice, the resulting precipitate was collected on Millipore HA filter paper (0.45µm). The filters were washed twice with trichloroacetic acid and then with water after which they were allowed to dry. The radioactivity was determined by liquid scintillation counting using a Beckman LS3801 liquid scintillation counter. This was done by adding 3 mL of Aqueous Counting Solution (ACS) to the filters in counting vials and then counting the radioactivity.

In vitro synthesis assay was carried out by incubating 69 μL of enzyme fraction in the presence of 1.3 mM of each of the constituent amino acids of CyA (with the exception of MeBmt and D-alanine), 1 mM DTE, 12.8 mM $MgCl_2$, 6.4 mM ATP, 0.1 M Tris-HCl, pH 7.5, 3.7 mM S-adenosyl methionine and 2 μM (0.1 μCi) ^{14}C-leucine for a total of 30 minutes. The reaction was stopped by adding 2 mL of water and the cyclosporin formed extracted with 2 mL ethyl acetate. 500 μL of ethyl acetate layer was then mixed with 5 mL of toluene fluor and radioactivity counted as above.

2.8. Isolation of chromosomes:

2.8.1. Preparation of chromosomal DNA:

Chromosomes were prepared by growing the cultures for 48 hours using the method described in 2.3. The contents of the 40 flasks were then aseptically filtered through 8 layers of cheese cloth to collect the mycelia. Contaminating spores were removed by resuspending the mycelia in sterile distilled water and shaking the suspension at 150 rpm for 30 minutes. Mycelia were recollected by filtration through a new layer of cheese cloth and resuspended in 200 mL of protoplasting medium containing 10mM Tris-HCl, pH 7, 10mM $MgCl_2$, 1mM 2-mercaptoethanol and 0.8M $MgSO_4.7H_2O$. The cell walls were then removed by treatment with Driselase, a crude enzyme preparation containing laminarinase, xylanase and cellulase (Kyowa Hakko Kogya Co., Tokyo Japan). Driselase was added to the suspension at a concentration of 10 mg/mL and the mixture incubated at 27°C for 18 hours after which the digested mycelia were filtered again through cheese cloth. The resulting filtrate was centrifuged for 10 minutes at 1,500 x g in a Sorvall®RC-5B refrigerated superspeed centrifuge. The protoplasts, appearing as a creamy layer on top of the filtrate, were removed using a pasteur pippette and washed twice with protoplasting medium to remove any residual driselase. Finally the protoplasts were pelleted in 2x mannitol based medium containing 10mM Tris-HCl, 10mM $MgCl_2$, 1mM 2-mercaptoethanol and 0.5M mannitol. The protoplasts were then resuspended in 1M mannitol-50mM ethylene diamine tetraacetic acid (EDTA), pH 8 and added to an equal volume of 1% low melting point agarose at 50°C in the same buffer. The mixture was then dispensed into a mold chamber (Bio-Rad) and allowed to solidify at room temperature. The solidified blocks were then suspended is NDS buffer (0.01M Tris pH 8, 0.5M EDTA, pH 8 and 1% laurylsarcosine) containing 1 mg/mL of proteinase K and incubated for 18 hours at 50°C. To remove the NDS buffer and proteinase K, the blocks were washed three times in 0.05M EDTA, pH 8 for 15 minutes and stored in the same buffer at 4°C.

2.9.2 Pulse-field gel electrophoresis:

Chromosomes were separated by contour-clamped homogeneous electric field (CHEF) using the CHEF-DRII system (Bio-Rad). The gels were electrophoresed in 0.5x Tris-borate buffer (TBE: 45mM Tris base, 45mM boric acid, 1mM EDTA, pH 8) or 1x Tris-acetate buffer (TAE: 40mM Tris base, 40mM acetic acid, 1mM EDTA, pH 8) maintained at 12°C. DNA plugs were cut and applied in the wells of a gel formed by pouring 100 ml of molten Low Melting Point (LMP) or Low Electroendosmosis (EEO) agarose (Boeringer) in running buffer. Different agarose concentrations and running conditions were used. After running, gels were stained with ethidium bromide for 45 minutes and destained in distilled water for the same period. The gels were observed under UV light.

Chromosomal DNA from *S. cerevisiae* and *Schizosaccharomyces pombe* were used as molecular size reference markers.

3. RESULTS:

3.1 MUTAGENESIS AND SELECTION

In order to obtain mutants of *T. niveum* that produce more cyclosporin than their parent strains, UV light and epichlorohydrin were used. Selection for the desired trait was carried out using 4-aza-DL-leucine.

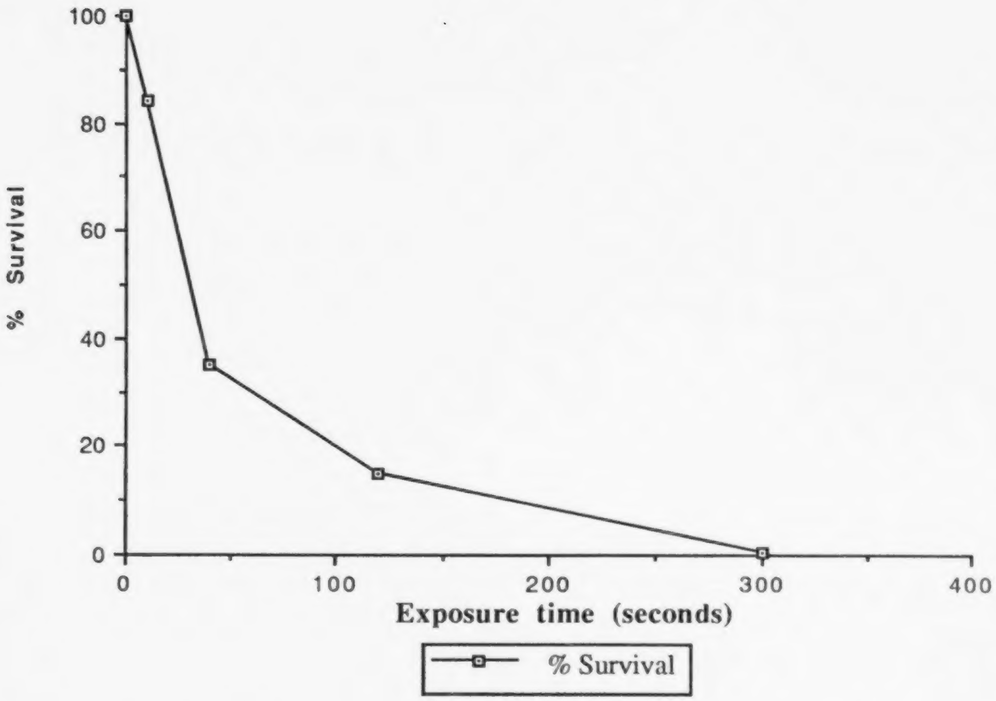
3.1.1 Determination of Optimum Conditions for Mutagenesis and Selection

The killing effect of UV light exposure was determined by exposing spores of *T. niveum* UAMH 4002 to UV light for 10, 40, 120 and 300 seconds. The irradiated spores were then serially diluted and plated on malt-yeast agar in triplicate. Colonies visible after 3 days of incubation were counted and used to determine the survival rate of the organism at the different levels of exposure. The desired kill rate of 99 % was obtained after 300 seconds of exposure (Figure 3) and was used in subsequent experiments for the isolation of UV mutants.

To determine the conditions required for obtaining a 99 % kill rate using epichlorohydrin, a 9 mL spore suspension of strain UV-98 containing 10^8 spores/mL was mixed with 1 mL of the mutagen to a final concentration of 0.15 M and incubated on a rotary shaker at 150 rpm and 27°C. This epichlorohydrin concentration was selected on the basis of the experiment conducted by Agathos and Parekh (1990) in which they obtained a 50% kill rate by exposing spores of *T. niveum* ATCC 34921 to 0.15 M epichlorohydrin for 150 minutes. At one hour intervals, 1 mL samples of the suspension were collected and the mutagen removed by washing the spores twice with sterile distilled water. The samples were then serially diluted, plated on malt-yeast extract agar in triplicate and incubated at 27°C. This experiment was not successful in that no growth was observed on all the plates other than the control for up to 14 days.

Figure 3: Survival of *T. niveum* UAMH 4002 spores after exposure to UV Light

Spores of strain UAMH 4002 were irradiated with UV light for 10, 40, 120 and 300 seconds. The spores were then serially diluted and plated onto malt yeast agar. Colonies appearing after 3 days of incubation at 28°C were counted and used to determine the % survival.The loss of viability appeared to follow one-hit kinetics.


% Survival
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80
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20
0
0
100
200
300
400
Exposure time (seconds)
% Survival

In order to estimate which concentration of epichlorohydrin would be appropriate for achieving a 99% kill rate, a second experiment was carried out using concentrations of the mutagen ranging from 0.001 M to 0.15 M and exposure periods of 1, 10, 30, and 100 minutes. Because of the large number of parameters involved, only single plate cultures were used for each condition. The kill rate was determined as explained above using plates incubated for six days. Although these results of this experiment were not very representative, they were able to show that the desired kill rate could be obtained by using epichlorohydrin concentrations between 0.01 M and 0.15 M using different exposure times. The 0.1 M concentration was finally selected for use in subsequent experiments. This was based on the fact that when very low concentrations of the mutagen (alkylating agent) are used in organisms that exhibit the adaptive response mechanism, induction of this mechanism at these mutagen concentrations leads to resistance of the organism to the mutagen. This factor was observed in this experiment by exposing the organism to 0.001 M of epichlorohydrin. The number of survivors at this concentration did not change after 10 minutes of exposure for a total exposure time of 100 minutes. In addition, even when resistance is not a factor, long exposure times of the organism are required when low mutagen concentrations are used. A concentration of 0.15 M epichlorohydrin was also useful in this case because of the very short time of exposure required which would make manipulations difficult. In this experiment for example, the survival rate after 1 minute of exposure to this concentration was 5 %. This experiment also showed no growth in all plates containing spores exposed for 100 minutes to epichlorohydrin concentrations between 0.05 M and 0.15 M an indication that strain UV-98 is more sensitive to epichlorohydrin than *T. niveum* ATCC 34921.

Since the results of the experiment just described were based on single plates, a further experiment to determine the optimum exposure time required to obtain a 99 % kill rate using 0.1 M epichlorohydrin was carried out. This was prompted by the fact that a follow up experiment using 0.1 M epichlorohydrin and exposure time of 30 minutes revealed a lower kill rate than that determined before from the above experiment. This final experiment therefore involved treatment of spores for 10, 30, 60, and 100 minutes. All samples were plated in triplicate and the results of this experiment are shown in Figure 4. A 99 % kill rate was observed by exposing the spores to the mutagen for 60 minutes while a 30 minute exposure led to a survival rate of 49 %. No growth was observed on samples treated for 100 minutes.

The concentration of 4-aza-DL-leucine required to inhibit growth of the organism was determined by incorporating the chemical directly into the growth medium. Analysis of triplicate plates of *T. niveum* UAMH 4002, containing concentrations of 4-azaleucine ranging from 0.1 mg/mL to 1 mg/mL indicated that the organism was sensitive to the analogue at concentrations as low as 0.1 mg/mL and its growth was completely inhibited by analogue concentrations of 0.4 mg/mL and higher. For strain UV-98 an almost complete inhibition of growth was observed in plates containing 4-azaleucine concentrations of 0.8 mg/mL (97.4 % kill rate) and 1 mg/mL (99.8 % kill rate). Mutants of *T. niveum* 4002 and UV-98 were therefore selected against 0.4 mg/mL and 0.8mg/L or 1 mg/mL of 4-azaleucine respectively.

3.1.2 Isolation and selection of mutants:

The isolation of mutants hyperproducing cyclosporins was initiated by exposing spores of *T. niveum* UAMH 4002 spores to UV light for 300 seconds as described in 2.6.1. The mutants were then selected by plating the irradiated spores on fructose-salt medium containing 0.4 mg/mL 4-azaleucine. The 103 isolated mutants were immediately tested for cyclosporin production by scraping off their mycelia from five day old master

Figure 4: Survival of spores of UV-98 after exposure to epichlorohydrin

Spores of strain UV-98 were mixed with epichlorohydrin to a final concentration of 0.1 M and incubated at 28°C on a shaker at 200 rpm. Samples of the suspension were collected 10, 30, 60 and 100 minutes and washed three times with sterile distilled water to remove the mutagen. The spores were then serially diluted and plated on Malt-yeast agar. Colonies appearing after 3 days of incubation at 28°C were counted and used to determine the % survival.

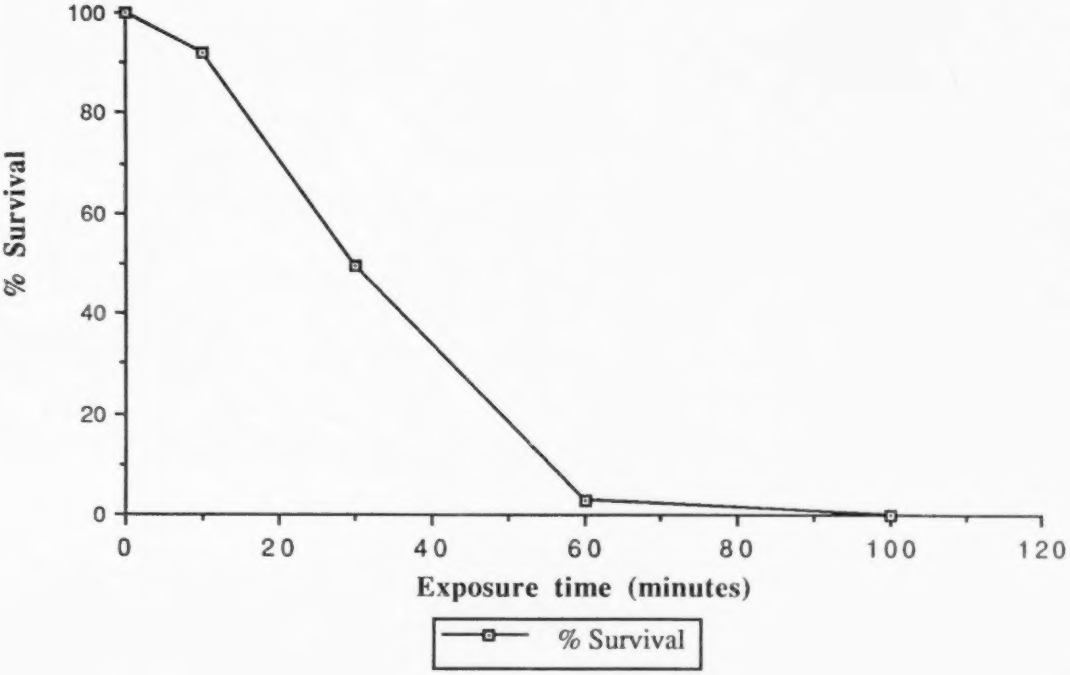
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Table 1: Production of cyclosporin by selected UV generated mutants

Strain	FINAL pH	Dry Cell Wt. g/L	Total Cy mg/L
UAMH 4002	4.3	5.7	120±11
UV-37	4.4	6.7	138±12
UV-74	4.4	6.0	128±2
UV-98	4.5	5.5	176±6

From the 103 mutants generated by UV induced mutagenesis, 3 strains producing the highest cyclosporin levels were selected and their cyclosporin levels determined using standardized inoculum i.e. 10^8 spores/mL. 10 mL of homogenized preculture of each strain were inoculated in triplicate into 100 mL of production medium containing L-sorbose as the carbon source and incubated at 27°C on a rotary shaker at 200 rpm. After 12 days of growth, the cultures were analysed for pH, mycelial dry weight and cyclosporin production.

The four highest producing mutants obtained after 30 minutes of epichlorohydrin treatment (EPI-68, EPI-70, EPI-74 and EPI-78) and three from the 60 minute treatment were further analysed for cyclosporin production using standardized inoculum and different carbon sources. The approach to include carbon source as a basis for selecting the highest producer was based on the results obtained with UV-98 in Table 3 and those obtained by Agathos and Parekh (1990) in which it had been shown that the carbon source showing highest cyclosporin production in the parental strain does not necessarily have the same effect on its mutants. The results of this experiment are shown in Table 2. Except for strains EPI-27 and 18, the rest of the strains showed higher (average) cycloporin production than UV-98 in both sorbose and maltose. The highest production was achieved by EPI-74 using maltose as the carbon source. This strain was therefore retained and used in subsequent experiments. Its selection was based on the average cyclosporin levels and reproducibility between the three samples used in the experiment.

3.1.3 Stability of mutants:

Throughout this research project, it had been observed that levels of cyclosporin produced by the mutants UV-98 and EPI-74 seemed to be decreasing with time thus leading to non-reproducible levels between different experiments. The levels of UV-98, for example had decreased from 176.25 mg/L to less than 120 mg/L towards the end of the project. The decrease in productivity by mutant strains was also observed by Fode (1990) in which the UV-generated mutants of T. niveum UAMH 2472, B-5 and B5E-84 lost productivity levels with time. Some of the factor cited as causes for decreased production were subculturing and storage conditions.

To determine if the storage conditions played a role in lowering the productivity of cyclosporin, spore stocks of UV-98 and EPI-74 were stored at 5°C, -20°C and -80°C. Under normal circumstances, spore stocks were maintained at -80°C. On a monthly basis for six months, the production of cyclosporin by the two mutants was monitored using

Table 2: Production of cyclosporin by selected epichlorohydrin generated mutants

Of the 138 epichlorohydrin generated mutants, seven were selected for further development. In this experiment the production of cyclosporin by the seven strains was compared to that of their parental strain using standardized inoculum of 10^8. Each of the strains was inoculated in triplicate in medium containing 2% w/v of sorbose, fructose or maltose. The cultures were incubated for 12 days and analysed for cyclosporin using the HPLC.

Strain	Carbon source	Final pH	Mycelial dry wt. g/L ±S.D.	Total Cy mg/L±S.D.
UV-98	Sorbose	4.1	5.4±0.1	89±2
	Maltose	4.2	5.0±0.1	99±9
	Fructose	4.1	4.8±0.1	115±12
EPI-78	Sorbose	4.1	5.8±0.1	99±8
	Maltose	4.2	5.3±0	114±22
	Fructose	4.1	4.5±0.1	100±14
EPI-68	Sorbose	4.1	5.8±0.2	105±13
	Maltose	4.2	5.3±0.2	108±5
	Fructose	4.1	4.6±0	116±8
EPI-61	Sorbose	4.1	5.9±0.1	98±5
	Maltose	4.2	5.07±0.3	122±12
	Fructose	4.2	4.49±0	109±27
EPI-74	Sorbose	4.2	6.3±0.1	104±4
	Maltose	4.3	5.4±0.5	128±0.3
	Fructose	4.2	5.4±0.2	88±6
EPI-70	Sorbose	4.2	6.7±0.2	92±8
	Maltose	4.3	6.3±0.2	117±8
	Fructose	4.2	5.9±0.7	112±3
EPI-27	Sorbose	4.2	4.7±0.2	69±9
	Maltose	4.3	4.9±0.4	83±7
	Fructose	4.2	4.0±0.1	89±6
EPI-18	Sorbose	4.2	4.9±0.2	82±0.7
	Fructose	4.2	3.8±0.2	95±4
	Maltose	4.3	3.9±0.4	121±3

spores stored at the three temperatures as inoculum. The results of this experiment are shown in Figures 5 and 6.

The production of cyclosporin by both mutants using spores stored at -80°C did not show any decrease with time. Instead, variations in production were observed between experiments, an indication that factors other than storage conditions of the spore stocks could be responsible for decreased cyclosporin production in the mutants. It is therefore possible that almost all the loss in productivity could be due to subculturing.

3.2 CHARACTERIZATION OF MUTANTS

3.2.1 The effect of carbon source on cyclosporin production:

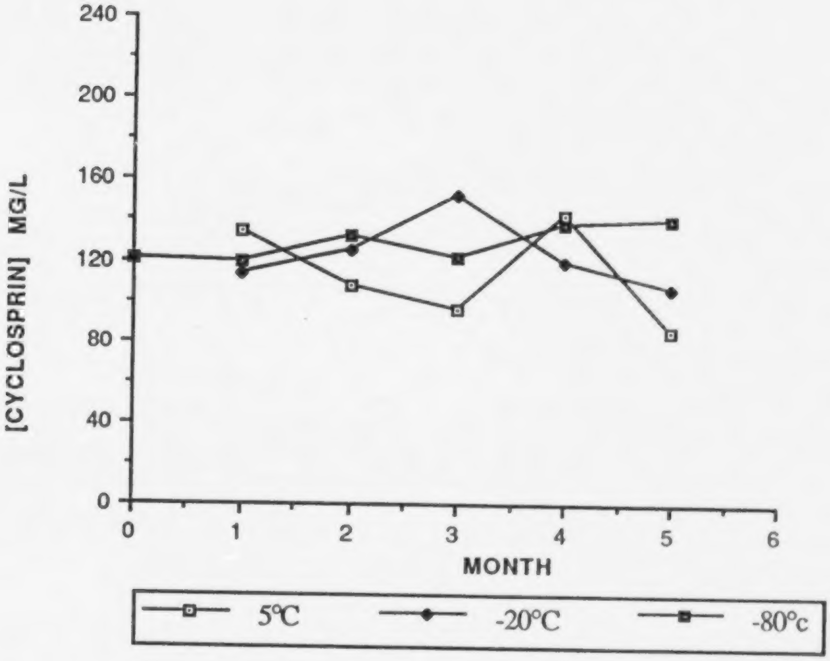
At the beginning of this project, available literature had shown that the production of cyclosporin could be supported by a number of various carbon sources (Agathos et al,1986; Isaac, 1988). It had also been shown that that the use of different carbon sources resulted in different rates and extents of production. At the time, sorbose had also been found to promote the best cyclosporin production in the strains tested. Experiments were therefore carried out to determine if sorbose or other carbon sources promoted the best cyclosporin production in the mutant strains.

The initial study to determine the effect of carbon source on cyclosporin production was carried out using mutant B-5, a UV mutant of *T niveum* UAMH 2472. The carbon sources used were sorbose, glucose, maltose, sucrose, fructose, galactose, ribose and lactose and were added to the production medium at a concentration of 2 % w/v. Cultures containing B-5 or UAMH 2472 were incubated for 12 days before analysing for cyclosporin production and biomass formation. The results of this experiment are depicted in Table 3.

The production of cyclosporin as well as growth of the organism was supported by all the carbon sources used in both strains with the exception of lactose in the wild strain. The highest biomass formation and production of cyclosporin were achieved in

Figure 5: The effect of cyclosporin production by UV-98 spore stocks stored at different temperatures

Spore stocks of UV-98 were dispensed in 1 mL aliquots and divided into three groups. Each group was then stored at a different temperature i.e. 5°C -20°C and -80°C. On a monthly basis, each group was tested for any changes in cyclosporin production in comparison to that obtained at the start of the experiment and also between the three groups.


240
200
160
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80
40
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[CYCLOSPRIN] MG/L
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1
2
3
4
5
6
MONTH
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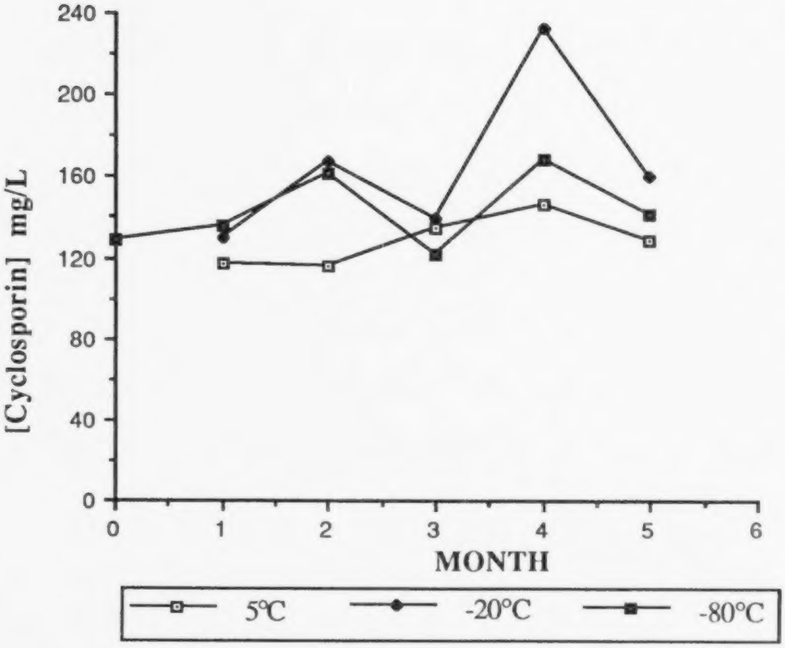
-20°C
-80°c

Figure 6: The effect of cyclosporin production by EPI-74 spore stocks stored at various temperatures

Spore stocks of EPI-74 were divided into three groups and stored at 5°, -20° and -80°C. Once a month for up to five months, the spore stocks were compared for cyclosporin production using maltose based production medium.


[Cyclosporin] mg/L
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200
160
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6
MONTH
5°C
-20°C
-80°C

Table 3. Production of cyclosporin by *T. niveum* UAMH 2472 and B-5 using various carbon sources

Preculture inoculum of *T. niveum* UAMH 2472 and B-5 grown on sorbose based medium were inoculated in triplicates into flasks containing sorbose, glucose, lactose, maltose, galactose, fructose, sucrose or ribose. Biomass and cyclosporin formation by these cultures were determined after 12 days of incubation.

Strain	**Carbon source**	**Final pH**	**Dry cell wt. g/L±S.D.**	**Total Cy mg/L±S.D.**
UAMH 2472	None	6.7	3.4±0.3	46±1
	Sorbose	5.2	10.5±0.6	164±7
	Glucose	5.0	9.7±0.5	133±20
	Lactose	6.3	4.5±0.5	46±17
	Maltose	5.3	9.0±0.3	148±6
	Galactose	5.3	8.6±0.2	125±2
	Fructose	5.3	9.9±0.3	134±3
	Sucrose	5.4	10±2	76±33
	Ribose	5.4	8.1±0.3	126±28
B-5	None	6.8	3.0±0.2	115±10
	Sorbose	5.5	10.4±0.3	387±18
	Glucose	5.2	8.9±0.1	386±18
	Lactose	6.0	6.4±0.7	206±43
	Maltose	5.5	8.8±0.6	349±47
	Galactose	5.6	8.1±0.7	266±16
	Fructose	5.5	10.3±0.0	267±74
	Sucrose	5.5	10.2±0.6	308±56
	Ribose	5.5	8.4±0.3	260±32

cultures grown in sorbose and the least in lactose. The rest of the carbon sources did not show any correlation between biomass formation and cyclosporin production. When compared to the parental strain, production of cyclosporin by the mutant was twice or more in all cases. The highest increases were observed with sucrose and lactose which produced four times and almost five times more cyclosporin than UAMH 2472 grown in the same medium. Cyclosporin production was also affected by pH of the media.

Two other experiments carried out along the same line involved comparing carbon source effect on cyclosporin production by UAMH 4002 , UV-98 and EPI-74. The carbon sources used were fructose, maltose and sorbose and were again added to the production medium at a 2 % w/v concentration. Biomass formation and cyclosporin production were analysed after 12 days of incubation and the results are shown in Table 4.

From the first experiment which involved UAMH 4002 and UV-98, it was observed that the three carbon sources did not have very different effects on volumetric cyclosporin production by UAMH 4002. The only major difference was in their levels of biomass in which sorbose showed the highest amount followed by maltose. The highest specific production of cyclosporin was achieved in maltose based medium followed by fructose. In the case of UV-98, highest biomass production was supported by maltose while fructose supported the highest metabolite production. Again no direct relationship could be derived between biomass formation and cyclosporin production, an indication that carbon sources highly favoured for biomass formation do not always provide the physiological state necessary for optimal secondary metabolite production.

In the other experiment involving UV-98 and its mutant EPI-74, it was again observed that of the three carbon sources used, fructose supported the highest production of cyclosporin by UV-98, despite the lower levels obtained in this study compared to the one just described. For EPI-74, maltose was the best carbon source for metabolite production followed by fructose while high biomass was achieved using sorbose.

Table 4: The Production of cyclosporin by UAMH 4002, UV-98 and EPI-74 cultures grown on different carbon sources

Flasks containing 100 ml of cyclosporin production medium containing sorbose, maltose or fructose were inoculated in groups of three with 10 mL of preculture suspensions of UAMH 4002, UV-98 or EPI-74. The cultures were then incubated for 12 days after which cyclosporin production and biomass formation were analysed. Two separate experiments, A and B, were conducted.

A:

Strain	Carbon source	Final pH	Dry cell wt. g/L ± S.D.	Total Cy mg/L ± S.D.
UAMH 4002	Sorbose	4.3	6.5±0.1	94±9
	Maltose	4.3	4.7±0.3	96±12
	Fructose	4.4	5.3±0.3	97±12
UV-98	Sorbose	4.5	4.6±0.3	125±21
	Fructose	4.3	5.4±0.3	186±12
	Maltose	4.4	6.0±0.4	160±4

B:

UV-98	Sorbose	4.1	5.4±0.1	89±2
	Maltose	4.2	5.0±0.1	99±9
	Fructose	4.1	4.8±0.1	115±12
EPI-74	Sorbose	4.2	6.3±0.1	104±4
	Maltose	4.3	5.4±0.5	128±0
	Fructose	4.2	5.4±0.2	88.25±6

A further experiment aimed at defining the carbon concentration best suited for cyclosporin production by UAMH 4002, UV-98 and EPI-74 was carried out. The carbon source investigated for each strain was one which had shown highest metabolite production in the preceding experiments and the concentrations compared were 1%, 2% and 5% w/v. The results of this experiment (Table 5) showed that increasing the carbon concentration led to increases in biomass production in all cases except UAMH 4002 grown in 2% fructose. Cyclosporin production on the other hand was not statistically different between the different concentrations especially in EPI-74 where the levels between 2% and 5% carbon source were the same and only marginally different from those obtained using 1% maltose. The amount of cyclosporin produced by EPI-74 in this experiment was however significantly higher than that of UV-98 compared to the results depicted in Table 4. For UV-98, higher average levels of cyclosporin were obtained using 5% fructose but these were again not different from those obtained using the other two concentrations of fructose when the error margins are considered. The amount of cyclosporin produced by UV-98 was for the first time also lower compared to that of UAMH 4002 in 2% fructose. The reasons for this could not be determined because even if a decrease in productivity has been observed in this strain, its production has always remained higher than the parental strain. From these results, it was also rather difficult to decide which concentration would be appropriate for cyclosporin production by a particular strain but from an economic point and comparison of the specific productivities, 2% fructose would be appropriate for UAMH 4002 while a 1% carbon concentration would be appropriate for both UV-98 and EPI-74.

3.2.2 The effect of Nitrogen source on cyclosporin production

Another study which was carried out using mutant B-5 but not other mutants was to determine the effect of ammonium sulfate and Delcase™, a commercial complex

Table 5: The effect of carbon concentration on cyclosporin production

Preculture inocula grown for 72 hours in fructose (UAMH 4002 and UV-98) or maltose (EPI-74)based media were inoculated to production flasks containing the same media at carbon source concentration of 1, 2 and 5% w/v. The cultures weret hen incubated for 12 days before analysing for biomass formation and cyclosporin production. The final pH of each culture was also noted.

Strain/Carbon	% Carbon source	Final pH	Dry cell wt.g/L ± S.D.	Total Cy mg/L ± S.D.
UAMH 4002	1	4.5	4.4±0.5	93±2
Fructose	2	4.0	4.3±0.3	126±9
	5	4.1	6.1±0.1	131±6
UV-98	1	4.8	3.8±0.4	118±13
Fructose	2	4.0	4.9±0.4	122±7
	5	4.0	6.2±0.1	144±14
EPI-74	1	4.3	5.9±0.2	167±8
Maltose	2	4.1	6.8±0.4	170±11
	5	4.1	8.2±0.2	170±11

nitrogen source, as alternative nitrogen sources. The use of inorganic nitrogen sources such as ammonium and nitrate salts had proved to be unsatisfactory for cyclosporin production in *T. niveum* ATCC 34921 (Agathos *et al.* 1986). To investigate if this characteristic was strain specific, ammonium sulfate was added to the production medium replacing vitamin assay casamino acid (VACA) in the experimental cultures. The results of this study are shown in Table 6.

The production of cyclosporin by mutant B-5 was very low compared to other experiments. When compared with cultures containing ammonium sulfate however, it was observed that total cyclosporin production was reduced 8-fold in the presence of inorganic nitrogen thus confirming previous results that inorganic nitrogen does not support cyclosporin production. Fungal growth in ammonium sulfate cultures was also lower than the one grown in VACA containing medium.

The use of Delcase as an alternative source of nitrogen was carried out by replacing VACA in the production medium. The experiment was carried out using *T. niveum* 2472 and B-5 and the results are depicted in Table 7. Good fungal growth was observed in both. Delcase and VACA grown cultures in both strains. Replacement of VACA with Delcase also proved to be superior for cyclosporin production in UAMH 2472 cultures but not B-5. The effect of Delcase on cyclosporin compared to that of ammonium sulfate further confirmed the suitability of complex organic nitrogen medium components for cyclosporin production over inorganic ones.

3.2.3 The effect of Amino acids on the production of cyclosporin

The supplementation of amino acids to production medium has been one approach used to increase bioactive peptide synthesis including cyclosporin. In addition, the technique has been used to determine the role of individual amino acids in the synthesis of a particular metabolite. In cyclosporin production the use of amino acid

Table 6: The effect of ammonium sulfate on cyclosporin production

Nitrogen source	Final pH	Dry cell wt, g/L	Total Cy mg/L
$(NH_4)_2SO_4$	3.2	4.4±0.5	18±3
VACA	4.8	7.7±0.7	143±13

A preculture inoculum of *T. niveum* B-5 was grown in VACA containing medium for 72 hours. A 10% inuculum was then made into 100 mL production medium containing 10 g/L VACA or ammonium sulfate as the nitrogen source and incubated for 12 days after which the production of cyclosporin was determined.

Table 7: The production of cyclosporin using Delcase as nitrogen source .

Strain	Nitrogen source	Dry cell wt. g/L±S.D.	Total Cy mg/L±S.D.
UAMH 2472	VACA	10.3±0.1	127±7
	Delcase	8.8±0.4	302±11
B-5	VACA	9.2±0.3	367±10
	Delcase	7.1±0.7	258±1

Precultures of UAMH 2472 and B-5 grown in production medium containing vitamin assay casamino acids as the nitrogen soure were inoculated into production medium containing Delcase or vitamin assay casamino acids as nitrogen source. The cultures were incubated for 12 days and then analysed for cyclosporin.

supplementation has had different effects on the strains tested, an indication that strain variabilities occur.

In this study, the effect of amino acids on cyclosporin production by UAMH 4002, UV-98 and EPI-74 was tested using both constituent and non-constituent amino acids.In the initial experiment, five amino acids, four of which are part of the cyclosporin molecule, were investigated for their effect on cyclosporin production using the three strains. The amino acids L-leucine, L-valine, glycine, L-alanine and L-asparagine were each added to cyclosporin production medium at a concentration of 8 g/L. The experiment was carried out in triplicate and incubated for 12 days. The amounts of cyclosporin produced are shown in Table 8. Since amino acid supplementation has been shown to direct synthesis of specific cyclosporin variants in some cases, the individual cyclosporins produced in each instance are also shown. The results however do not show all the types of cyclosporins that may be formed as a result of supplementation due to lack of internal standards for these variants and also because no unusual peaks (other than Cys A, B, and C) were observed in this particular experiment unless their elution time from the HPLC column was greater than 10 minutes.

Except for valine supplemented medium, the exogenous addition of amino acids lowered the production of cyclosporin in both UAMH 4002 and UV-98. The addition of alanine in all the strains led to a reduction in total cyclosporin and directed synthesis of cyclosporin B, a metabolite not normally produced by these strains. Cyclosporin B is formed by the replacement of 2-aminobutyrate of CyA with L-alanine and in this case, its formation was done at the expense of both cyclosporins A and C. Directed synthesis of cyclosporin B was also observed in glycine supplemented medium in all the strains. In the wild type strain, addition of glycine did not only reduce total cyclosporin levels but also completely inhibited the formation of cyclosporin C. In UV-98 both total cyclosporin and cyclosporin C levels were reduced while in EPI-74 total cyclosporin was unaffected but both CyA and C were reduced to allow formation of CyB.

Table 8: Cyclosporin production in medium supplemented with amino acids

The various amino acids were added individually to cyclosporin production medium at a concentration of 8 g/L. The flasks each containing 100 mL of medium were then inoculated with 10 mL of preculture inoculum of UAMH 4002, UV-98 or EPI-74 and incubated for 12 days. The samples were assayed for cyclosporin production by HPLC. Strain EPI-74 was grown in a maltose based medium while UV-98 and UAMH 4002 were grown in fructose medium.

Strain/ Amino acid	Mycelial dry wt.g/L	Total Cy mg/L	% individual of Total Cy		
			Cy A	Cy B	Cy C.
UAMH 4002					
Control	4.6±0	68±5	75	0	25
L-alanine	3.9±0.4	51±2	70	17	13
L-asparagine	3.7±0.5	47±2	77	0	23
Glycine	3.8±0.2	44±3	78	22	0
L-leucine	4.3±0.3	70±9	80	0	20
L-valine	3.7±0.4	98±6	98	2	0
UV-98					
Control	4.1±0.3	116±16	72	0	28
L-alanine	4.6±0.4	89±7	66	17	17
L-asparagine	4.0±0.2	71±10	73	0	27
Glycine	3.8±0.4	67±2	75	14	11
L-leucine	3.6±0.1	78±15	78	0	22
L-valine	3.5±0.1	151±12	93	7	0
EPI-74					
Control	5.1±0	123±3	73	0	27
L-alanine	4.5±0.8	118±5	58	21	21
L-asparagine	4.1±0.3	122±5	56	22	22
Glycine	4.5±0.7	132±10	62	22	16
L-leucine	5.0±0.1	139±2	58	19	23
L-valine	4.3±0.1	182±5	88	12	0

The addition of asparagine, a non-constituent amino acid of cyclosporin did not show any effect on production of CyA and C in UAMH 4002 and UV-98 but for unknown reasons led to a decrease in total cyclosporin levels. In EPI-74 on the other hand the presence of asparagine did not have any major effect on total cyclosporin in that the control levels (123 mg/L) were essentially identical to those supplemented with asparagine (122 mg/L). L-Asparagine also led to directed synthesis of CyB mostly at the expense of CyA in this strain and to a lesser extent CyC. The addition of L-leucine to production medium of UAMH 4002 had no effect on total production or distribution of the individual molecules. In UV-98, leucine suppressed total production by about 30%: this result was somewhat suprising in that UV-98 was believed to leucine-deregulated and would therefore be expected to show increased cyclosporin production in the presence of this amino acid. The factors that could be responsible for this effect of leucine will be explained later in the discussion together with any other unusual effects observed with the other amino acids. In EPI-74, the addition of L-leucine increased total cyclosporin, directed the synthesis of CyB and suppressed Cy A and C synthesis.

Supplementation of production medium with L-valine led to an increase in total cyclosporin and an almost exclusive formation of cyclosporin A in all the three strains. The formation of cyclosporin B was also observed in all strains while CyC production was completely inhibited. No directed synthesis of cyclosporin D was observed (contains L-valine in position 2) .

As a result of the unique behaviour of L-valine i.e. formation of Cy A at the expense of almost all other homologs, an experiment to determine the effect of various concentrations of valine on cyclosporin production was carried out. Cyclosporin A is the most active of the cyclosporins so far tested and is the one currently used in immunosuppressive and other clinical work involving cyclosporins. A system supporting exclusive formation of this metabolite would therefore be desirable. Flasks containing 100 mL of production medium supplemented with 4 g/L, 8 g/L or 16 g/L of L-valine were inoculated with preculture inoculum of the three strains. The results obtained after 12 days incubation are shown in Table 9.

Although an increase in total cyclosporin production was observed in all strains, at all valine concentrations, increasing the amount of this amino acid did not improve metabolite formation in UAMH 4002 and UV-98. Instead, high formation of cyclosporin was observed in cultures containing 4 g/L valine, an indication that there was saturation in both strains and also a negative effect on cyclosporin production in UV-98 at high valine concentrations. For EPI-74, total cyclosporin increased marginally with increases in valine concentration probably demonstrating higher tolerance of the amino acid compared with its parental strain.

3.2.4 Growth of the organism and cyclosporin production

In order to establish the relationship between growth and production of cyclosporin production, time course experiments for each strain were carried out. These experiments were also used to try and establish the basis for high cyclosporin production in the mutants in comparison with their parent strains.

The growth curve for mutant B-5 was carried out by growing cultures of this organism in production medium containing sorbose as the carbon source. On a daily basis for 16 days, three flasks were removed from the shaker and analysed for pH changes, biomass formation and cyclosporin production. The results of this experiment are depicted in Figure 7. A rapid increase in biomass was observed during the first five days of growth followed by a slow growth up to day 12. In the case of cyclosporin production, an initial two day lag in production of the metabolite was observed following inoculation. Maximum cyclosporin yield was attained on day 12 thus coinciding with the highest biomass level. Beyond this point, both biomass and metabolite levels were reduced. The loss in biomass was most likely a result of hyphal fragmentation and lysis while the reason for cyclospoprin reduction is still unclear. Other than the decrease in cyclosporin

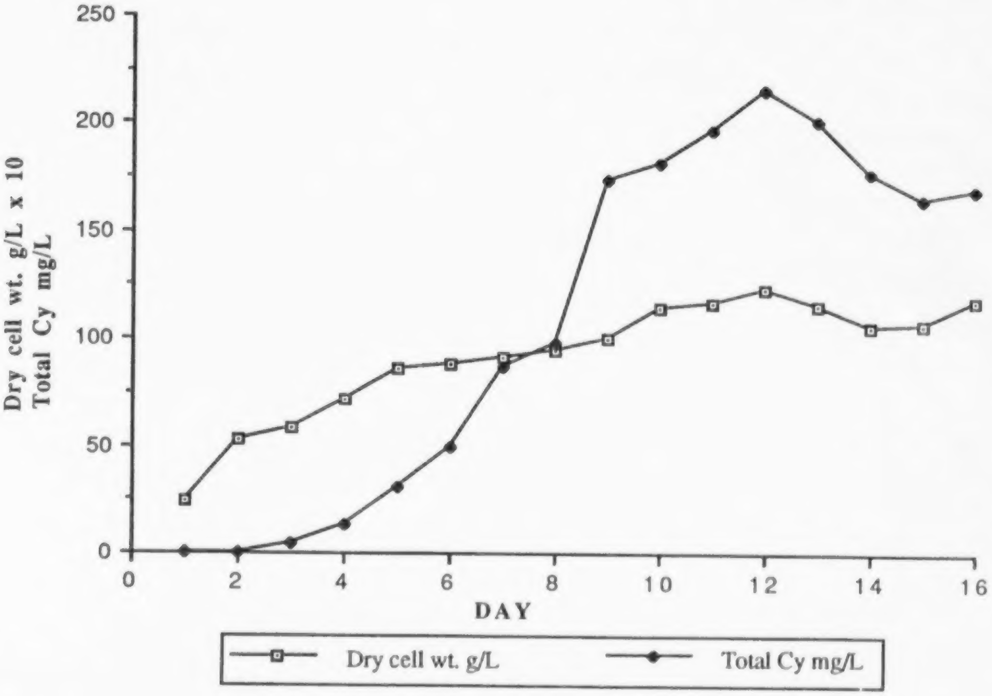
Table 9: Production of cyclosporin using different concentrations of L-valine

Production medium containing in addition 4g/L, 8g/L or 16 g/L L-valine were inoculated with 10 % preculture inoculum of each of the three strains and incubated for 12 days after which cyclosporin prodution, pH of the medium, and dry cell weight were determined.

Strain	L-valine g/L	Final pH	Dry cell wt. g/L ±S.D.	Total Cy mg/L ±S.D.
UAMH 4002	0	4.0	4.3±0.3	126±9
	4	4.3	5.1±0.2	180±2
	8	4.3	4.9±0.3	176±23
	16	4.6	5.7±0.3	175±21
UV-98	0	4.0	4.9±0.4	122±7
	4	4.2	5.0±0.1	217±9
	8	4.4	4.5±0.1	163±13
	16	4.8	5.2±0.2	184±11
EPI-74	0	4.1	6.8±0.4	170±11
	4	4.3	7.2±0.1	260±19
	8	4.3	7.1±0.1	264±21
	16	4.3	7.2±0.1	291±28

Figure 7: Growth and Production of Cyclosporin over Time in Shake Flask Cultures of mutant B-5

Five flasks containing 100 mL of sorbose-based production medium were each inoculated with 10^8spores of mutant B-5 and incubated for 72 hours. The cultures were then pooled, homogenized and 10 mL inoculated into each of the forty-eight flasks containing the same medium used above. On a daily basis for 16 days, three flasks were harvested and analysed for biomass formation and cyclosporin production.


Dry cell wt. g/L x 10
Total Cy mg/L
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
DAY
Dry cell wt. g/L
Total Cy mg/L

levels after day 12 the general pattern of growth and production in this organism were similar to those observed in its parental strain *T. niveum* UAMH 2472 (Isaac *et al.* 1990) and in ATCC 34921 (Agathos *et al.* 1986).

The relationship between growth and cyclosporin production in *T. niveum* UAMH 4002 and its mutant UV-98 were determined by analysing similar triplicate samples on a daily basis for 12 days. Both strains were grown in sorbose-based production medium. Production of cyclosporin was detected on day 1 in the wild strain and day 2 in the mutant. In both cases, rapid increases in biomass production were observed during the first four days followed by a slower growth phase up to day 8 and day 12 in the mutant and wild strain respectively. The slow growth phase in both strains was associated with rapid cyclosporin production. In UAMH 4002 maximum cyclosporin production was attained on day 12, which coincided with maximum biomass. In UV-98 on the other hand, a loss in biomass was observed after day 8 but cyclosporin production continued reaching a maximum on day 12. The results of this study are depicted in Figures 8a and 8b.

A comparison of growth and cyclosporin production between the two strains indicated that although biomass formation was similar in the these organisms, both producing a maximum of 6 g/L, UV-98 showed a higher rate of cyclosporin production compared to its parental strain (Figure 9). The specific production in this strain continued to increase even after the biomass had started decreasing in contrast to UAMH 4002 in which the biomass remained constant after day 7 of the fermentation at 16 to 20 mg/g. The behaviour of UV-98 was also in contrast to the results obtained by Isaac (1988) for *T. niveum* UAMH 2472 in which it was shown that as biomass increased, so did cyclosporin production, a condition observed with UAMH 4002.

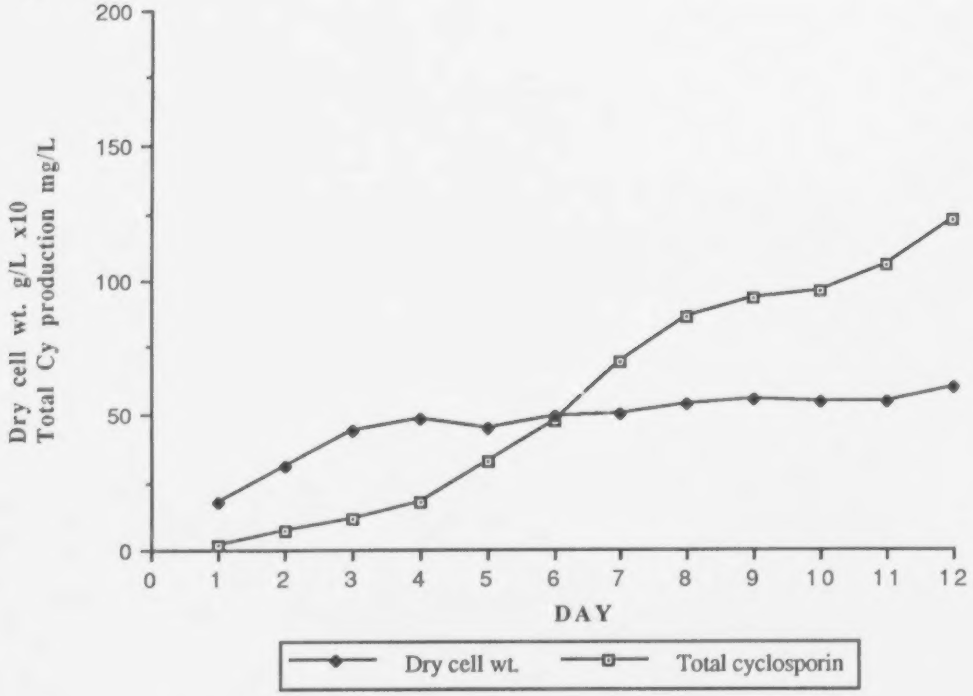
The growth curve for EPI-74 was determined by growing the organism in maltose based medium and analysing the cultures for cyclosporin production and biomass formation daily for 12 days. The results of this experiment are depicted in Figure 10.

Figures 8: Growth and Production of Cyclosporin over Time in Shake Flask Cultures of UAMH 4002 and UV-98

8a. Four flasks containing 100 mL sorbose based production medium were each inoculated with 10^8 spores of *T. niveum* 4002 and incubated for 72 hours. The cultures were then pooled, homogenized and 10 mL inoculated into each of the thirty-six flasks containing the same medium used above. Triplicate flasks were harvested daily and analysed for biomass formation and cyclosporin production.

8b. Spore inoculum of UV-98 was used and the experiment was carried out as explained for UAMH 4002

8a


200
150
100
50
0
Dry cell wt. g/L x10
Total Cy production mg/L
0
1
2
3
4
5
6
7
8
9
10
11
12
DAY
Dry cell wt.
Total cyclosporin

8b

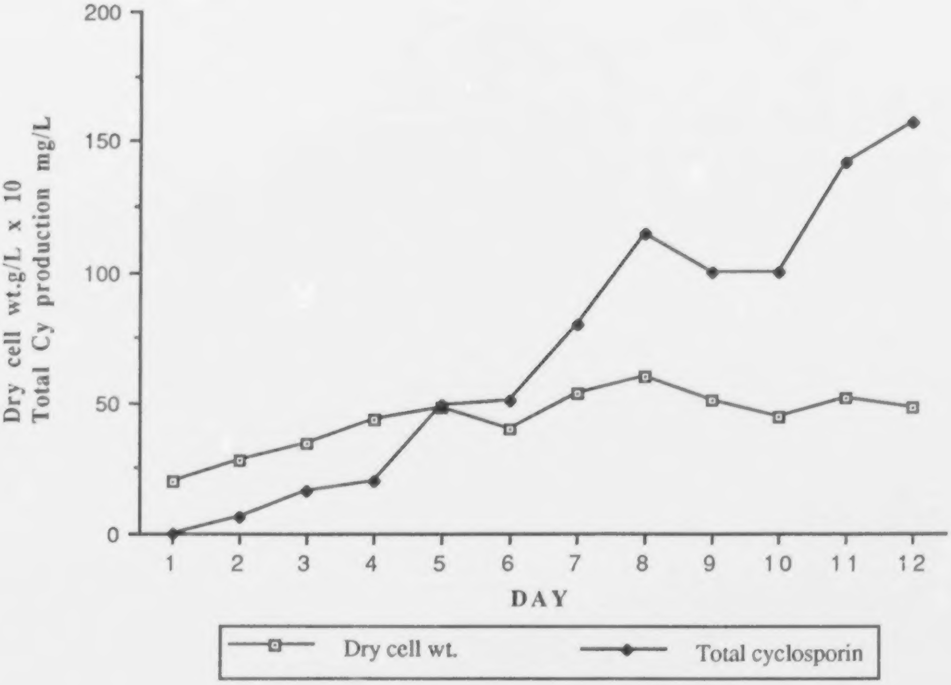

200
150
100
50
0
Dry cell wt.g/L x 10
Total Cy production mg/L
1
2
3
4
5
6
7
8
9
10
11
12
DAY
Dry cell wt.
Total cyclosporin

Figure 9: Comparison of Mycelial Dry Weight and Specific Cyclosporin Production in Shake Flask Cultures of *T.niveum* UAMH 4002 and UV-98

The data for this graph was obtained from the cultures described in Figure 8.

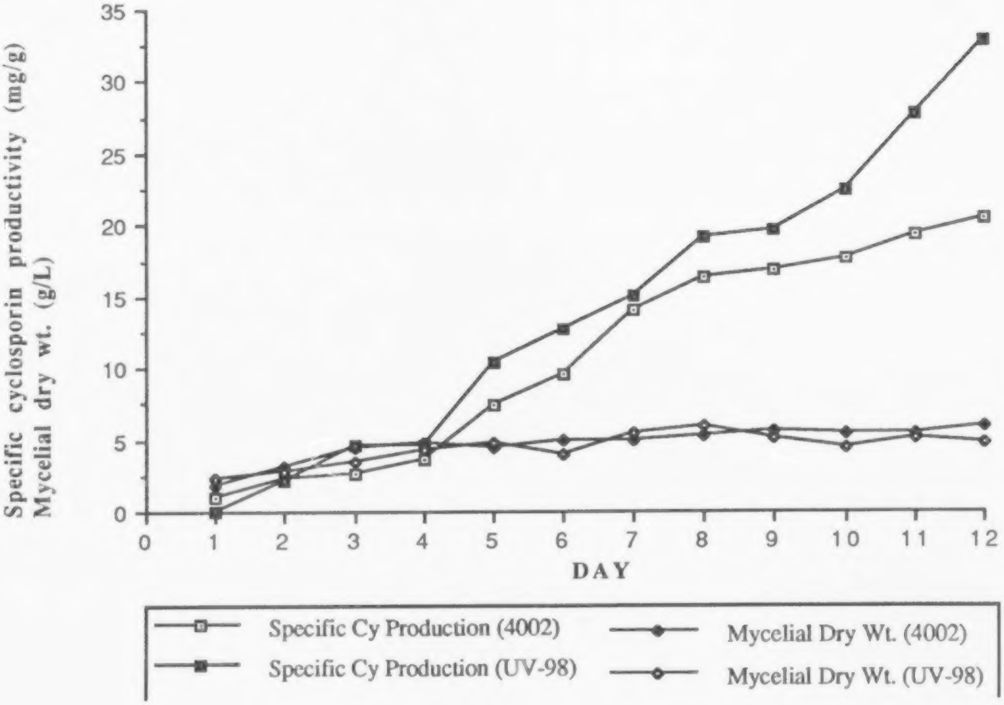
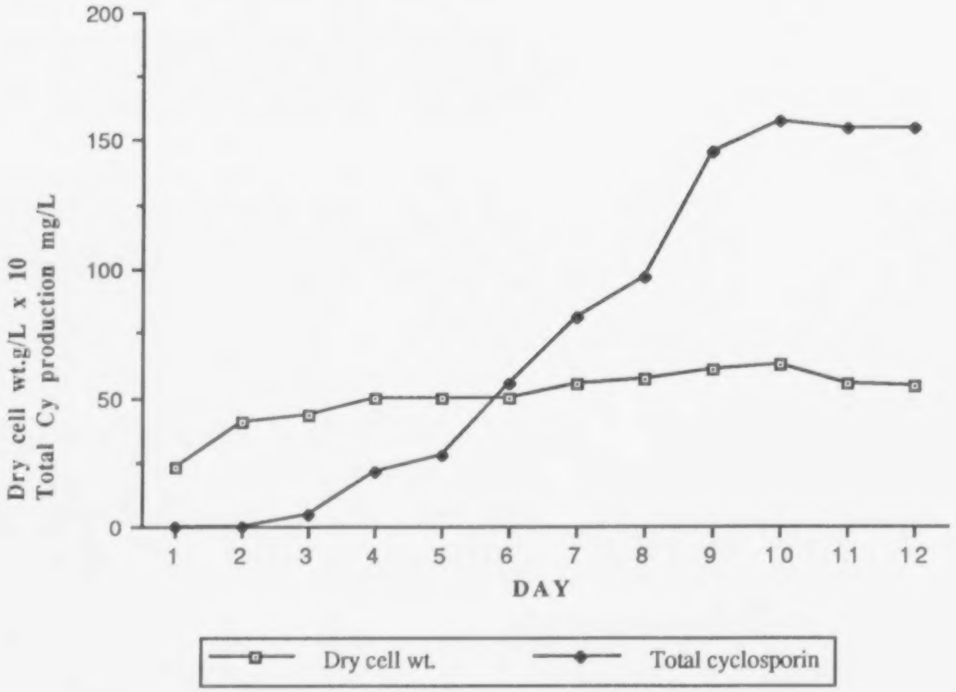

Specific cyclosporin productivity (mg/g)
Mycelial dry wt. (g/L)
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
9
10
11
12
DAY
Specific Cy Production (4002)
Specific Cy Production (UV-98)
Mycelial Dry Wt. (4002)
Mycelial Dry Wt. (UV-98)

Figure 10: Growth and Cyclosporin Production over Time in Shake Flask Cultures of EPI-74

Four flasks containing 100 mL of maltose-based production medium were each inoculated with 10^8 spores of EPI-74 and incubated for 72 hours. The preculture were then pooled, homogenized and 10 mL inoculated into a total of thirty-six flasks. On a daily basis,triplicate samples were analysed for biomass formation and cyclosporin production.


200
150
100
50
0
Dry cell wt.g/L x 10
Total Cy production mg/L
1
2
3
4
5
6
7
8
9
10
11
12
DAY
Dry cell wt.
Total cyclosporin

Rapid biomass formation was observed during the first four days followed by a slow growth phase up to day 10. Cyclosporin production was not detected until day 3 of fermentation. Thereafter, rapid production was observed reaching a maximum of 157 mg/L on day 10. Unlike its parent strain, the specific production of cyclosporin remained constant after day 8 of growth at 24 to 28 mg/g and in agreement with the results obtained with other strains of *T. niveum,* cyclosporin production increased with increasing biomass.

Another study which was carried out along with the growth curves was to determine if there was any relationship between cyclosporin production and morphology of the strains. Following mutagenesis, Chang and Elander (1979) showed that many high producing strains of *Penicillium chrysogenum* and *Chrysosporium acremonium* exhibited decreased vegetative vigour and/or reduced sporulation on plate cultures. The reduced mycelial vigour associated with high metabolite productivity was attributed to the presence of a highly efficient mycelial system. Recently, Agathos and Parekh (1990) also reported high sporulation and morphological differences between M6, an epichlorohydrin mutant of ATCC 34921. To determine if any of these were a factor in cyclosporin production by the mutant strains, *T. niveum* UAMH 4002, UV-98 and EPI-74 were grown on malt-yeast agar, Czapek dox agar and cyclosporin production medium incorporated with agar. On a daily basis, the colonies were observed for extent of growth, shape and arrangement of conidiophores, phialides and spores, mycelial texture and sporulation. These parameters were also determined during growth in liquid cultures using cyclosporin production medium.

No relationship could be established between enhanced cyclosporin production and morphology of the mutants from these studies. The radial growth of both the wild strain and mutants was similar in each of the three types of media. Highest growth was observed on malt-yeast agar and least in cyclosporin production medium. Also, no morphological, colonial or cultural differences were observed between the strains both on solid and liquid media. Sporulation in liquid cultures was observed on day 2 of fermentation and day 5 on solid media.

3.3 PURIFICATION AND IDENTIFICATION OF CY SYNTHETASE(S)

This study was carried out to determine how many enzymes are involved in the synthesis of cyclosporin. In their study, Lawen and Zocher (1990) showed that cyclosporin was produced by a single multienzyme isolated as a single band on denaturing SDS-PAGE. This was in contrast to the results of Fode (1990) in which, using a different purification method, it was possible to show the presence of up to four bands all of which may be involved in cyclosporin synthesis. Since no other independent study was available to determine whether the above results were due to strain variability or differences in methodology, experiments aimed at identifying the enzymes involved in cyclosporin synthesis were carried out using strains UV-98 or EPI-74 and comparing both methods of purification.

3.3.1 Extraction and purification of Cy synthetase(s)

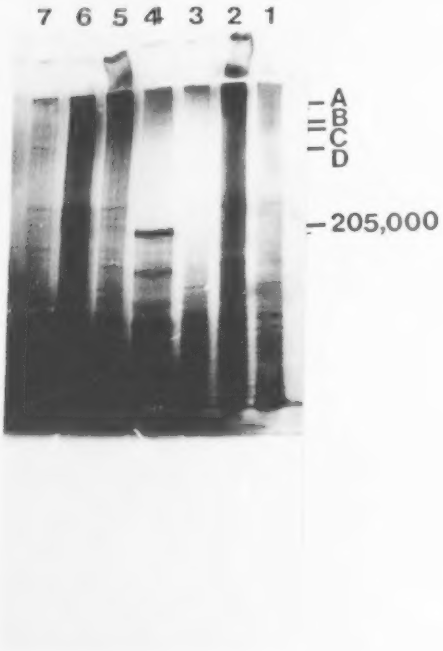
Cell-free extracts were prepared as described in section 2.7.1 and the proteins separated using SDS-PAGE. The results of this experiment are shown in Plates 1a and 1b. Analysis of the protein profile in the two gels showed the presence of 3 to 4 prominent bands designated A, B, C and D, in the region where Cy synthetase(s) are expected to occur. A comparison of the intensity of the four bands following silver staining showed that most of the protein was confined to band D. A minimal estimation of the molecular weights of the four proteins relative to those of myosin (205,000), ß-galactosidase (116,250) and phosphorylase b (97,400) showed protein A to have a molecular weight of 5.0×10^5. The molecular weights of proteins B, C and D were estimated to be 4.7×10^5, 4.4×10^5 and 4.1×10^5 respectively (Figure 11). In the study done by Fode (1990), the molecular weights of the four proteins were estimated to be between 3.8×10^5 and 4.8×10^5.

Plate 1: Separation of cyclosporin synthetase(s)

Crude extracts of mutant UV-98 were treated with PEI to precipitate the nucleic acids, followed by fractionation of the proteins using ammonium sulfate. The ammonium sulfate fractions were then desalted and subjected to gel filtration chromatography. Samples collected at each stage of purification were analysed by SDS-PAGE using 3.5% to 6% gradient gels. The proteins were applied to the gel at a concentration of 100 μg per lane.

1a: The proteins in this photograph were purified following the method of Lawen and Zocher (1990). Lane 1 represents the crude extract, lane 2: PEI treated extract, lanes 3, 5 and 6: protein precipitating between 0-20%, 30-50% and 20-30% ammonium sulfate respectively, lane 4: molecular weight markers and lane 7: 20-30% ammonium sulfate fraction after gel fractionation.

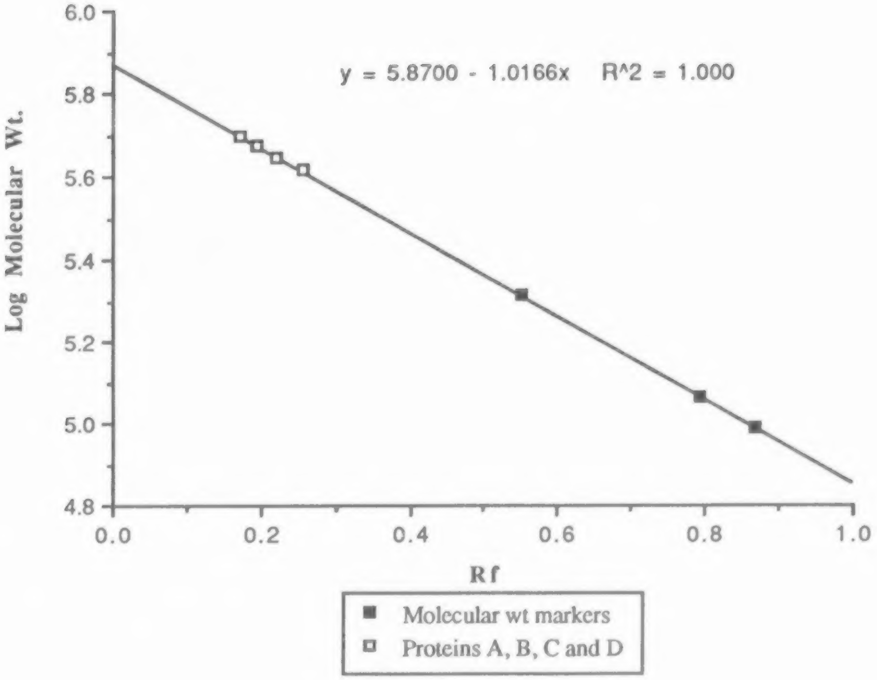
1b: The proteins were separated following the method used by Fode (1990). Lane 1: crude extract, lane 2: PEI treated extract, lanes 3, 4 and 6 represent ammonium sulfate fractions between 0-20%, 20-35% and 35-55%, lane 5: markers, and lanes 7, 8 and 9 represent 0-20%, 20-35%, and 35-55% ammonium sulfate fractions subjected to gel filtration.


7 6 5 4 3 2 1
A
B
C
D
205,000

9 8 7 6 5 4 3 2 1
A
B
C
D
205,000

Figure 11: Estimation of the molecular weight of cyclosporin synthetase(s)

The molecular weights of proteins A, B, C and D were estimated by plotting the Rf values of myosin, ß-galactosidase and phosphorylase b against the log molecular weights of these compounds. The Rf values were calculated using Plate 2a gel. The molecular weights of the proteins were determined using the equation shown in the graph.


y = 5.8700 - 1.0166x R^2 = 1.000
Log Molecular Wt.
6.0
5.8
5.6
5.4
5.2
5.0
4.8
0.0
0.2
0.4
0.6
0.8
1.0
Rf
■ Molecular wt markers
□ Proteins A, B, C and D

3.3.2 Detection of Cy synthetase

In order to determine which of these protein bands were involved in cyclosporin synthesis, experiments aimed at detecting the activity of the enzymes were carried out as described in section 2.7.4 in the presence of ^{14}C-leucine. It was hoped that once enzyme activity is detected in the case of the ^{14}C amino acid binding assay, then SDS gels of the reaction mixture would be carried out and the labelled protein identified by autoradiography. In the case of the *in vitro* synthesis assay, the approach was to separate the reaction products using thin layer chromatography followed by autoradiography. Such an experiment would provide information as to whether the purified enzyme was capable of synthesizing cyclosporins. Several attempts aimed at detecting the activity of the enzymes using cell-free extracts purified by the two methods mentioned above were unsuccessful, an indication that the extracts were probably inactive.

To maintain activity of the enzyme during purification, reducing agents are normally added to the buffers and they also form part of the reaction mixture for any assays aimed at detecting the enzyme activity. In this study, the reducing agent used was dithioerythreitol (DTE) but it was not until the time of preparing this thesis that it was learnt that the chemical supplied to our laboratory as DTE was actually ß-lactoglobulin. Because of the time period required to expose the autoradiographs (8 weeks), it was not possible to repeat these experiments and unless this is done is is not possible to state which of the four proteins are involved in the synthesis of cyclosporin.

3.4 ISOLATION OF CHROMOSOMES OF *T. NIVEUM*

The last objective of this research project was to establish an electrophoretic karyotype of *T. niveum*. In order to do this, a methodology for obtaining intact genomic DNA and separation of this DNA into individual chromosome bands had to be developed.

3.4.1 Preparation of intact genomic DNA

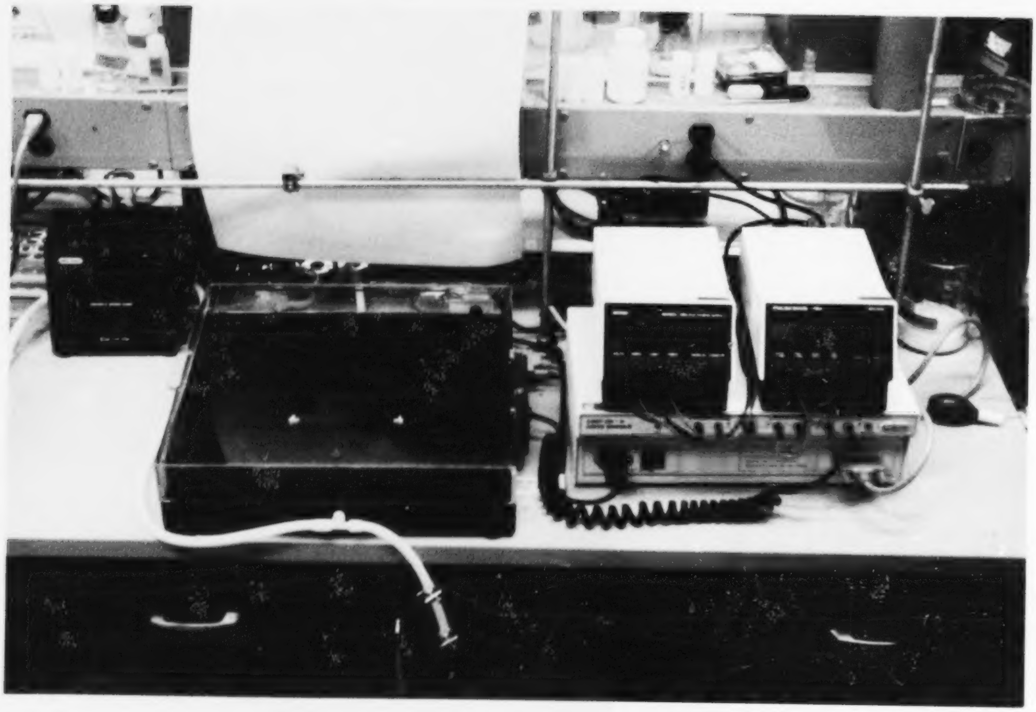
Intact chromosomal DNA was prepared by digesting mycelial cells of the organism with 15 mg/ml Driselase in 0.8 M $MgSO_4$, 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7 for 18 hours at 28°C. The resulting protoplasts were obtained through centrifugation of the digested filtrate and harvested in 2x mannitol solution as described in section 2.8.1. The presence of protoplasts was determined by observing the filtrate under the microscope. The protoplasts appeared as large circular structures about 5 times larger than the conidia of *T. niveum*, and, in most cases clumped together thus making it difficult to determine their concentration. The amount of DNA obtained from these protoplasts was also not determined due to limited amounts of protoplasts obtained. The intact DNA was embedded in agarose by mixing the protoplasts in 1 M mannitol-50 mM EDTA, pH 8 with an equal volume of molten agarose in the same buffer so that the final concentration of agarose in the plugs was 0.5 %. The DNA was released from the protoplasts by suspending the plugs in NDS buffer (described in section 2.8.1) containing 1 mg/ml proteinase K and incubating overnight at 50°C. Complete lysis was observed by the sinking of the plugs to the bottom of the test tube. The plugs were then washed 3 times with 0.05 M EDTA for 15 minutes and left overnight in the same buffer at room temperature. The plugs were stored at 4°C in 0.05 M EDTA, pH 7.5. Attempts to prepare the protoplasts using other enzymes (e.g. glusulase and lyticase) were less successful.

3.4.2 Separation of chromosomes

The separation of the DNA into individual chromosomes was achieved using pulse field gel electrophoresis, a technique capable of physically separating DNA molecules ranging from 50-12,000 kilobases in size. In this case, contour-clamped homogeneous electric field (CHEF) electrophoresis (Plate 2) was used, using low melting point (LMP) or low electroendosmosis (EEO) agarose with concentrations ranging from 0.6 to 1%. The running buffers were 0.5x TBE or 1x TAE.

Plate 2:.The CHEF Apparatus

The photograph shows the buffer tank (with gel) which consists of 24 electrodes arranged in a hexagonal array and offers reorientation angles of 120°.



Due to lack of literature on chromosome size of this organism, initial attempts to separate the chromosomes were carried out using various conditions including those used for the separation of the chromosomes of *Saccharomyces cerevisiae*, *Candida albicans* and *Neurospora crassa:* some 10 runs of 2 to 10 days were carried out during these exploratory experiments. It was not until the conditions described by Brody and Carbon (1989) for the separation of *Aspergillus nidulans* chromosomes were used that some form of separation was observed. Using these conditions, which included running of gels in 0.5x TBE buffer (instead of 0.5x TAE used by Brody and Carbon) at 45 V and three pulse intervals of 50 minutes, 45 minutes and 37 minutes at durations of 72 hours, 12 hours and 72 hours respectively, two bands were observed. The gel concentration used in this study was 0.8% LMP agarose. In order to improve the mobility and separation of the bands, lower gel concentration of 0.6 and 0.7 % were used in subsequent experiments. The voltage, temperature and pulse times were not changed except that longer running periods were used for each pulse interval with total running time ranging between 10 to 14 days. These experiments were, however, unsuccessful for a total of six runs in that there was smearing of bands in all samples, including the *S. pombe* and *S. cerevisiae* markers from BioRad. Attempts to use the conditions for separation of *S. pombe* which included running 0.6% gels at 50V for a pulse interval of 60 minutes for 160 hours were also unsuccessful. At the time that these problems were going on, it was also observed that the cooling system was mulfunctioning in that it would at times freeze the running buffer in the coil thus preventing circulation. On more than one occassion, the CHEF apparatus automatically turned off the power but it could not be determined if this was related to the cooling system.

Since the cause of lack of separation could not be determined some changes were made to the type of buffer and agarose used. A system using 1xTAE buffer and 0.8 % EEO agarose was devised. Although TAE buffer had not been used in most of the running periods involved which would lead to its deterioration, it was at this point assumed that since very low electric current was being used the buffer would sustain for a longer period of time. It was also assumed that since migration of DNA is faster in this buffer than TBE, the amount of time required to run the gels would be reduced. There were no specific reasons for changing the type of gel. The gels were run at 12°C and 50 V at pulse intervals of 50 minutes, 45 minutes and 37 minutes for 48 hours, 12 hours and 24 hours respectively. The results of this experiment are depicted in plate 3a. From this experiment , total separation of the three *S. pombe* marker chromosomes was achieved and *T. niveum* was separated into four bands, one of which looks at least like a doublet. In order to try and separate the *T. niveum* bands further, another experiment was carried out using the same conditions as above but run for a total period of seven days. The results of this experiment are shown in plate 3b. Increasing the total running time improved the separation of the *T. nivuem* chromosomes. The two bands migrating as a doublet were separated with one band appearing faintly in position two from the top. The total number of chromosomes was therefore determined to be at least five. It should be noted that at the time these two experiments were carried out, the cooling system had been repaired and therefore the observed separation of the chromosomes may not necessarily be due to changes in the type of gel and running buffer composition, although the use of TAE buffer reduced the total time required for separation of the chromosomes.

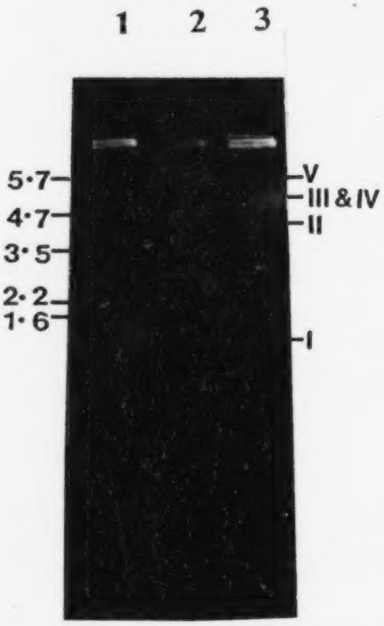
3.4.3 Estimation of the sizes of *T.niveum* chromosomes

The sizes of the chromosomes were estimated by comparing their mobility on the gel with that of *S. pombe* molecular weight markers (Figure 12). A plot of the mobilities of the *T. niveum* chromosomes with those of the *S. pombe* markers revealed a linear relationship except for band V whose molecular weight was estimated using the *S. cerevisiae* markers. From Figure 10, the molecular weights of the *T. niveum* chromosomes were estimated to be 5.6 Mb (V), 5.0 Mb (III andIV) and 4.5 Mb (II). The molecular weight of band I was estimated to be 1.2 Mb. From these experiments, it could not however be determined if band I represents a chromosome or is mitochondrial DNA. The total genome size of *T. niveum* as determined from these experiments should therefore be between 20.1 and 21.3 Mb.

Plate 3: Separation of Chromosomal DNA of *T. niveum* by CHEF

3a. The agarose gel was electrphoresed for a total period of 84 hours using 0.8 % EEO agarose and 1x TAE buffer. The pulse times used were 50 minutes for 48 hours, 45 minutes for 12 hours and 37 minutes for 24 hours. Lane 1, *S. cerevisiae*; lane 2, *T.niveum* (UV-98); lane 3, *S. pombe* (972).

3b. The gel was run for a total of 168 hours using the same agarose and buffer conditions as in 3a. The same pulse times were used with running times of 72 hours, 24 hours and 72 hours. Lanes 1-3 represent different protoplast preparations of *T. niveum;* lane 4, S. *pombe;* lane 5, *S. cerevisiae.*


1
2
3
5·7
4·7
3·5
2·2
1·6
V
III & IV
II
I

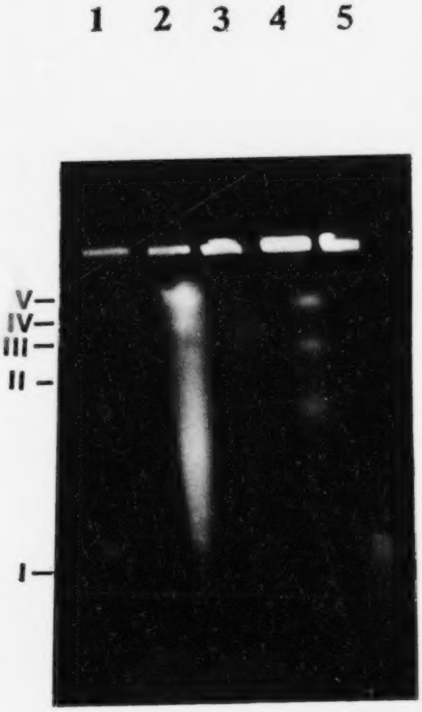
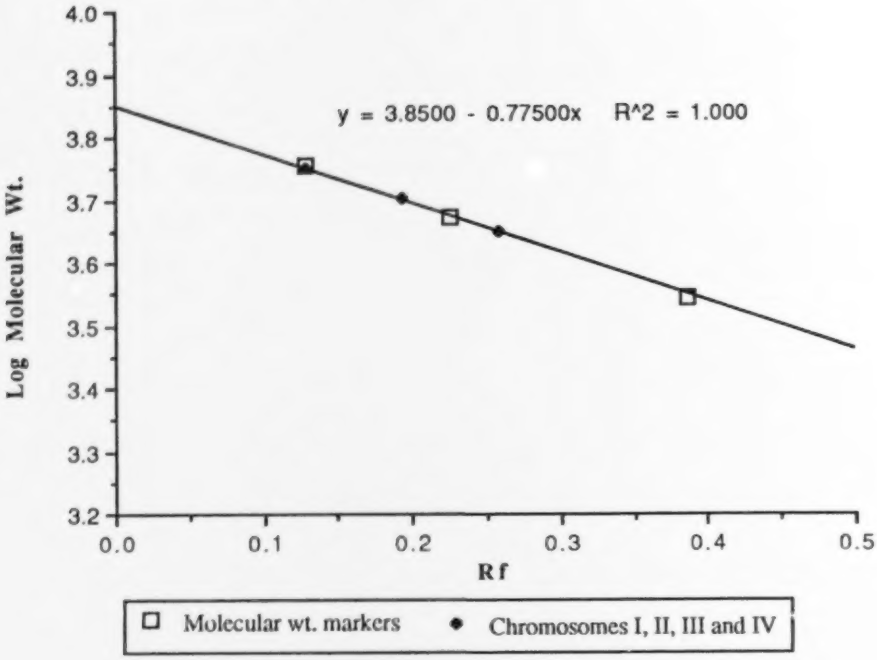

1
2
3
4
5
V
IV
III
II
I

Figure 12: Estimation of the molecular size of *T. niveum* chromosomes

The molecular sizes of bands I-V were estimated by plotting the log molecular weights of the three chromosomes of *S. pombe* 972 (5.7 Mb, 4.7 Mb and 3.5 Mb) against the Rf value. Using the relationship established from this curve, the molecular weights of the *T. niveum* chromosomes were estimated. The smallest band was however outside of the range with these markers and was therefore estimated using the *S. cerevisiae* markers which, for the same reason could not be incorporated into this graph.


y = 3.8500 - 0.77500x R^2 = 1.000
Log Molecular Wt.
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
0.0
0.1
0.2
0.3
0.4
0.5
Rf
□ Molecular wt. markers
● Chromosomes I, II, III and IV

4. DISCUSSION

One of the objectives of this research project was to isolate mutants of *T. niveum* capable of producing higher cyclosporin titres using UV-light and epichlorohydrin. The effects of carbon source, nitrogen source and amino acid supplementation on cyclosporin production by some of these mutants were investigated and studies aimed at determining the number of enzymes involved in cyclosporin production were carried out. In another aspect of this project, chromosomes of *T. niveum* were also isolated as an initial step directed towards the understanding of the genetic makeup of this organism.

4.1 MUTAGENESIS AND SELECTION

Tolypocladium niveum mutants capable of producing high cyclosporin titres were obtained using UV light and treatment with epichlorohydrin. In both cases, a rational selection procedure was used based on the resistance of the mutants to 4-azaleucine, an analogue of the amino acid leucine. Although the amino acid analogue used may be best chosen based on the knowledge of production of the rate-limiting precursor amino acid(s), the lack of this information for cyclosporin production led to the use of 4-azaleucine. This choice was based on the premise that L-leucine is the most abundant amino acid in the cyclosporin molecule and therefore the chances of its production being limited may be high.

Using UV light induced mutagenesis and selection with 4-azaleucine, mutant UV-98 was obtained. This mutant was capable of producing 47 % more cyclosporin that its parent strain UAMH 4002. A second round of mutagenesis involved the treatment of UV-98 with epichlorohydrin and led to the isolation of strain EPI-74. Although this strain had shown high levels of cyclosporin immediately following mutagenesis (along with the six other strains shown in Table 2) using isolated colonies as inocula, these levels were not observed when standardized inocula were used. Instead, levels almost the same as those produced by the original parental strain (UV-98) were observed. The reason for these lower values was not determined but the results obtained in Table 2 compared to those in Appendix 6.2 and 6.3 seem to suggest that the strains could have lost most of the productivity of cyclosporin following subculturing. Strain EPI-74 was selected based on the average values of total cyclosporin produced. Although not significant in all cases, EPI-74 continued to produce more cyclosporin than UV-98 in all the subsequent experiments.

A comparison of the effectiveness of the two mutagens in this study showed that UV light was more effective than epichlorohydrin for the generation of mutants capable of producing high cyclosporin titres.

An investigation of the stability of strains UV-98 and EPI-74 using spores stored in 20% glycerol at 5°C, -20°C and -80°C showed that the storage temperature did not play any significant role in lowering the productivity of cyclosporin observed in these mutants. The observed loss in productivity could have been due to subculturing. Although the problem of instability among mutants has been recognized for a long time, its genetic basis is still unclear. It has been suggested that some of the causes may be partial duplication of certain chromosomes, diploidy or aneuploidy and unstable mutations (Peberdy, 1988).

4.2 CHARACTERIZATION OF MUTANTS

4.2.1 The effect of carbon source on cyclosporin production

In studying the production of cyclosporin by wild strains of *T. niveum* using different carbon sources, Agathos *et al.* (1986) and Isaac *et al.* (1990) found sorbose to be the best carbon source for cyclosporin production. Later, Agathos and Parekh (1990) showed that cyclosporin production by strain M6, an epichlorohydrin generated mutant was best supported using maltose as the carbon source.

An investigation of the carbon sources in this project showed that both fungal growth and cyclosporin production were supported by several carbon sources. For strain

UAMH 2472, highest biomass and cyclosporin production were observed using sorbose. This was in agreement with the results obtained by Isaac *et al.* using the same strain. Similar results were obtained for its mutant B-5 but in this case both sorbose and glucose and to some extent maltose supported the highest production of cyclosporin. The least production by B-5 was observed with lactose while in UAMH 2472 this carbon source was not metabolised. In contrast to the results of Isaac *et al.*, sorbose did not give the highest titre with strain UAMH 4002. Instead, production was found to be similar using any of the three carbon sources tested with sorbose producing the highest amount of biomass (Table 4). The reason for this contrast was not determined. In strains UV-98 and EPI-74, fructose and maltose supported the highest production of cyclosporin respectively.

The results from these experiments suggest that the carbon source supporting highest cyclosporin yield is strain specific and for maximum metabolite production to be realized an assessment of the best carbon source should be carried out. An example of the importance of this factor is shown in Table 4a in which using fructose, the amount of cyclosporin produced by strain UV-98 was 94% more than that of UAMH 4002 compared to the initial 47% produced using sorbose (Table 1). It must be noted that cyclosporin production was not always reproducible between different experiments and therefore comparisons between strains were made based on the data of individual experiments. Increasing the concentration of the best carbon source did not increase the cyclosporin levels in strains UAMH 4002, UV-98 and EPI-74, an indication that there could be some control of metabolite production by the carbon source used. Biomass production was increased with an increase in carbon concentration indicating that the level of cyclosporin production is not always related to the amount of biomass formed.

4.2.2 The effect of nitrogen source on cyclosporin production

The use of ammonium sulfate as a nitrogen source for cyclosporin production by strain B-5, a UV generated mutant of strain UAMH 2472 (Fode, 1990) was investigated. Inorganic nitrogen sources such as ammonium and nitrate salts had proved to be unsatisfactory for cyclosporin production in *T. niveum* ATCC 34921 (Agathos *et al.* 1986). Substituting ammonium sulfate for vitamin assay casamino acids in the growth medium showed that cyclosporin production by strain B-5 was suppressed 8 fold in the presence of ammonium sulfate. This was so even though the total cyclosporin levels of strain B-5 were low in this particular experiment. Replacement of VACA with Delcase, another complex nitrogen source reduced the production of cyclosporin by 30% in B-5 while an increase of 139% was observed with strain UAMH 4002. These two experiments confirm the observation that cyclosporin production is best supported using complex nitrogen sources. Also, the amount of cyclosporin produced by a particular strain may be dependent on the nitrogen source used.

4.2.3 The Effect of amino acid supplementation on cyclosporin production

The effect of adding amino acids to cyclosporin production medium was initially investigated by Kobel and Traber (1982). In this study, it was possible to show that cyclosporin production could be increased or directed towards a specific form through the addition of specific amino acids in the growth medium. These observations were confirmed by Agathos *et al.* (1989) and Isaac *et al.* (1990) and in addition, their results suggested that the effect of a particular amino acid on cyclosporin production may be strain specific. Exogenous addition of amino acids has been reported to increase metabolite production in other peptide metabolites. By adding valine and methionine to production media, Madry *et al.* (1983) and Drew and Demain (1975) were able to show increased enniatin and cephalosporin C production respectively.

In this research project amino acid supplementation was carried out using L-valine, L-alanine, L-leucine, glycine and L-asparagine. In all the three strains tested, addition of L-alanine led to directed synthesis of cyclosporin B, but also suppressed total cyclosporin production in strains UAMH 4002 and UV-98 (Table 8). This was in agreement with the results of Kobel and Traber but not with Isaac *et al.* in which an increase in total cyclosporin was observed in all the strains tested. Directed synthesis of cyclosporin B was also obtained through the addition of glycine in all the strains. The substitution of glycine for D-alanine has been reported in the *in vitro* synthesis of cyclosporin (Lawen and Zocher, 1990). Whether this nonchiral amino acid can also substitute for L-alanine in the cycloporin structure is not known but from these results it is possible. Again, total cyclosporin production was suppressed in strains UAMH 4002 and UV-98 in the presence of glycine while EPI-74 was unaffected. The reduction in cyclosporin was higher in both strains, 35% to 42% in the presence of glycine compared to 23% in the presence of alanine. The effect of glycine on UAMH 4002 and UV-98 was in agreement with the results of Agathos *et al.* (1986) in which they showed that glycine had no effect on cyclosporin A production in semi-synthetic medium.

The addition of L-valine to production medium increased total cyclosporin production in all strains and there was an almost exclusive formation of CyA. These observations were in agreement with the results of the other studies cited above, except those obtained by Kobel and Traber in which directed synthesis of CyD was observed and valine did not support the formation of CyA. The reason for the directed synthesis of cyclosporin A of valine instead of cyclosporin D in the presence was not be determined, but this could be related to the way valine is metabolized in the cell. Cyclosporin A consists of 2-aminobutyric acid in position 2, a compound analogous to L-valine. This amino acid (2-aminobutyric acid) has been shown to compete with valine in bacterial systems. Although this competition has not been reported in fungal systems, it may well occur in this instance. Increasing the amount of exogenously supplied valine did not lead to an increase in cyclosporin production in all the strains. An amount of 4 g/L or less would therefore be appropriate for supplementation.

The suppresion of total cyclosporin by L-asparagine (a non-constituent amino acid) in strains UAMH 4002 and UV-98 and its ability to direct CyB formation in UV-74 was an indication of the low specificity of the enzymes involved in cyclosporin synthesis. Although the role of L-asparagine was not determined, its behaviour in this experiment was an indication that the synthesis of cyclosporin can be negatively affected even by molecules which are not part of its normal structure. This characteristic has been reported by Traber *et al.* (1989) in which they showed that exogenous addition of DL-3-aminobutyric acid, a compound not incorporated in the cyclosporin molecule, suppressed the formation of cyclosporin.

Exogenous addition of L-leucine had no effect on UAMH 4002 but a reduction of 32% in total cyclosporin of strain UV-98 was observed in contrast to the anticipated results whereby the presence of leucine was expected to increase the cyclosporin levels. The reasons for the failure of this amino acid to support cyclosporin production in this strain were not determined but it may suggest that the organism was not leucine-deregulated and that the observed increase in cyclosporin titres could be due to other factors. For a precursor to be incorporated efficiently into a molecule of a secondary metabolite however, it must be adequately supplied and stored in the cells of the organism. Also, the precursor must not be metabolized in some other pathway. In *E. coli,* growth of the organism with exogenously added leucine has been found to suppress the transport of leucine into the cells (Quay *et al.* 1975). While inhibition of the uptake of leucine could have played a role in affecting the levels of cyclosporin in strain UV-98 it is still unclear why similar results were not observed with the other two strains.

Of all the amino acids used to supplement cyclosporin production medium, only L-valine had a similar effect on both production of individual cyclosporins and total metabolite production by all the three strains (Table 8), an indication that the effects may be strain specific. On most occasions, cyclosporin production by a particular amino acid was affected in a similar pattern in strains UAMH 4002 and its mutant UV-98. The conclusion from this experiment is that by supplementing cyclosporin production medium with constituent amino acids, production towards certain forms may be directed but this does not always lead to increased titres except in the case of valine. Also, since CyA is the most important form used clinically so far, future work should be directed at increasing its titres. This may be achieved by supplementation of production medium with valine in those strains where the addition of this amino acid favours the formation of CyA. Alternatively, mutants deregulated in valine feedback control should be investigated for their effect on CyA production.

4.2.4 Growth of the organism and cyclosporin production

The relationship between growth of the organism and cyclosporin prodution over time were determined by measuring the biomass and amount of cyclosporin produced in liquid cultures on a daily basis. Morphological and cultural differences between the strains were also sought. In all the strains investigated, except UAMH 4002, cyclosporin production was not observed until day 2 of the fermentation which coincided with the beginning of sporulation. In some *Penicillium* species grown on solid media, Campbell *et al.* (1982) showed that secondary metabolism began only after aerial mycelia had started forming. The delay in cyclosporin production until spore formation might therefore indicate the importance of sporulation in the production of cyclosporin. Some mutants of *T. niveum* unable to produce cyclosporin also have been shown to be incapable of sporulation (Fode, 1990).

In general, biomass measurements of the different strains showed that there was rapid growth of the organisms during the first four days after which biomass continued to increase slowly up to day 12. Maximum cyclosporin production was achieved between day 4 and day 12. These results were in agreement with those reported for other strains of *T. niveum*.

The failure to detect any morphological differences between strains UAMH 4002, UV-98 and EPI-74 on both solid and liquid media was an indication that the ability to produce high cyclosporin levels in the mutants must be related to high rate of metabolite production. This was shown with UV-98 (Figure 7) in which the specific production of cyclosporin by this mutant was higher than that of UAMH 4002 despite the fact that the two strains produced similar amounts of biomass.

4.3 PURIFICATION AND IDENTIFICATION OF CYCLOSPORIN SYTHETASE(S)

The goal of this study was to determine the number of enzymes involved in the biosynthesis of cyclosporin. Using the purification methods described by Lawen and Zocher (1990) and those used by Fode (1990), four proteins which may be involved in the synthesis of cyclosporin were separated under denaturing SDS-PAGE (Plate 1). The estimated molecular weight of these proteins designated protein A, protein B, protein C and protein D were 5.0×10^5, 4.7×10^5, 4.4×10^5 and 4.1×10^5 daltons respectively. These sizes were close to those obtained by Fode for the four proteins separated following SDS-PAGE. Lawen and Zocher on the other hand reported that cyclosporin synthetase is a single polypeptide chain with an estimated molecular weight of 8.0×10^5 and is separated as a single band with a molecular weight of 6.5×10^5 daltons on 3% denaturing SDS-PAGE.

Using the results shown in Plate 1, it was not possible to determine which of the four proteins are involved in cyclosporin production due to lack of data on enzyme activity. It was therefore not possible to explain the presence of four proteins in this system as compared to one in the other. It is also not shown in the results of Lawen and Zocher whether or not the band identified as cyclosporin synthetase was the only band present in this molecular weight range or it was the only band capable of *in vitro* synthesis of CyA and activation of ^{14}C labelled amino acids. Studies aimed at determining whether cyclosporin synthetase occurs as a multienzyme polypeptide or as a multienzyme complex therefore need to be continued. This is necessary if cell-free systems are to be used as an alternative for cyclosporin A production or for the synthesis of novel cyclosporins which cannot be produced *in vivo*. The use of high cyclosporin producing strains may aslo prove more useful for such studies and if a good comparison of the molecular weights of the purified proteins is to be made, then high molecular weight markers have to be used. The largest molecular weight marker used in this project was myosin (205,000 daltons) compared with tyrocidin synthetase III (450,000 daltons) used by Lawen and Zocher.

4.4 ISOLATION OF CHROMOSOMES OF *T. NIVEUM*

Fungal protoplasts can be obtained by treating the mycelia or conidia with lytic enzymes capable of removing the cell walls. This is carried out in the presence of an osmotic stabilizer. In this research project, *T. niveum* protoplasts were obtained by treating the mycelia with 15 mg/mL of Driselase in the presence of 0.8 M $MgSO_4$ for a total period of 18 hours at 28°C. Chromosomal DNA was released from the protoplasts embedded and treated in agarose plugs. The processed agarose plugs were stored in 0.05 M EDTA, pH 7.5 and remained in good condition for over 7 months.

The separation of large DNA molecules is dependent on a number of factors, some of which include agarose concentration, buffer concentration, temperature, pulse times, voltage and total electrophoresis run time. Therefore, in order to separate the entire genome of *T. niveum* into individual chromosomes, various combinations of these factors were assessed. The *T. niveum* chromosomes were finally separated into four bands (one of which was migrating as a doublet (Plate 3a)) using 0.8% EEO agarose and pulse intervals of 50 minutes, 45 minutes and 37 minutes. Under these conditions, the three *S. pombe* chromosomes were also separated within 84 hours instead of 160 hours. By comparing the sizes of the *T. niveum* chromosomes with those of *S. pombe* and *S. cerevisiae*, the total genome size of *T. niveum* was determined to be between 20.1 and 21.3 Mb, a size bigger than that of *S. pombe* 972 (13.9 Mb) but smaller than *Aspergillus niger* (31 Mb) and *Aspergillus nidulans*(35.5-38.5). Attempts to separate the chromosomes further by extending the total running time seemed to separate the doublet but the band designated as II in Plate 3b was not clearly visible probably due to small DNA quantities. For this reason bands II and III were identified as a doublet with total size of 10 Mb. Further work should therefore be carried out using the running condtions described in Plate 3b and using agarose plugs with a high DNA content.

Further work also needs to be carried out on protoplast preparation methods and isolation of chromosomes. In this study the amount of protoplasts obtained following cell wall digestion was very little, making DNA quantitation difficult. A growth medium that would generate a lot of biomass within the period when DNA content in the cells is still high should be sought. Another factor which proved difficult during the study was the inability to determine the number of protoplasts obtained from the mycelia due to clumping together of the former. A knowledge of this would be helpful in determining the effectiveness of the enzyme in digesting a given amount of biomass. The use of TBE buffer and Low Melting Point agarose for the separation of chromosomes should also be investigated further to confirm their suitability for this purpose.

5. REFERENCES

Aarnio, T. and Agathos, S.N. 1989. Production of extracellular enzymes and cyclosporin by *Tolypocladium inflatum* and morphologically related fungi. Biotechnol. Lett. 11: 759-764.

Agathos, S.N. and Parekh, R. 1990. Enhancement of Cyclosporin Production in a *Tolypocladium inflatum* strain after epichlorohydrin treatment. J. Biotechnol. 13: 73-81.

Agathos, S.N., Marshall, J.W., Moraiti, C., Parekh, R. and Madosingh, C. 1986. Physiological and genetic factors for process development of cyclosporine fermentations. J. Ind. Microbiol. 1: 39-48.

Billich, A. and Zocher, R. 1990. Formation of N-methylated Peptide Bonds in Peptides and peptidols. In: Biochemistry of Peptide Antibiotics. Kleinkauf, H. and von Döhren, H. (eds). Walter de Gruyter. Berlin, p. 57-79

Billich, A. and Zocher, R. 1987. Enzymatic Synthesis of Cyclosporin A. J. Biol. Chem. 262: 17258-17259.

Bissett, J. 1983. Notes on *Tolypocladium* and related genera. Can. J. Bot. 61: 1311-1329.

Blackshear, R.J. 1984. Systems for polyacrylamide gel electrophoresis. Methods Enzymol. 104: 237-255.

Bradford, M.M. 1976. A rapid and sensitive method for the quanitification of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72: 248-254.

Brody, H. and Carbon, J. 1989. Electrophoretic karyotype of *Aspergillus nidulans*. Proc. Natl. Acad. Sci,.USA. 86: 6260-6263.

Byrne, G. 1988. Cyclosporin turns five. Science. 242: 198.

Campbell, I.M., Doerfler, B.A., Remaley, A.T., Rosato, L.M. and Davis, B. N. 1982. Secondary metabolism and colony development in solid cultures of *Penicillium brevicompactum* and *Peniccilium patulum*. In: Overproduction of Microbial Products. Krumphanzl, B., Sikyta, B. and Vanek, Z. (eds). Academic Press, New York. p141-152.

Dittmann, J., Lawen, A, Zocher , R. and Kleinkauf, H. 1990. Isolation and partial characterization of cyclosporin synthetase from a cyclosporin non-producing mutant of *Beauveria nivea*.. J. Biol. Chem. 371: 829-834.

Drew, S.W. and Demain, A.L. 1975. The obligatory role of methionine in the conversion of sulfate to cephalosporin C. Eur. J. Appl. Microbiol. 2: 121-128

Dreyfuss, M., Harri, E., Hoffman, H., Kobel, H., Pache, W. and Tscherber, H. 1976. Cyclosporin A and C. New metabolites from *Trichoderma polysporum* (Link ex Pers.) Rifai. Eur. J. Appl. Microbiol. 3:125-133.

Chang, L.T., and Elander, R.P. 1979. Rational Selection for Improved Cephalosporin C Productivity of *Acremonium chrysogenum* Gams. Dev. Ind. Microbiol. 20: 367-379.

Fliri, H.G. and Wenger, R. M. 1990. Cyclosporine: Synthetic Studies, Structure-activity relationships, Biosynthesis and Mode of Action. In: Biochemistry of Peptide Antibiotics. Kleinkauf, H. and von Döhren, H. (eds). Walter de Gruyter. Berlin, p. 245-287.

Fode, C. J. 1990. M.Sc Thesis. University of Alberta

Gams, W. 1971. *Tolypocladium.* Eine hyphomycetengattung mit geschwollenen phialiden. Persoonia 6: 185-191.

Hanawalt,.P.C. 1977. DNA repair processes: Overview. In DNA repair processes. Nichols, W.W. and Murphy, D.G. Symposia Specialists Inc. Miami. p. 1-19

Helland, D.E., Male, R., Haukanes, B.I., Olsen, L., Haugan, I. and Kleppe, K. 1987. Properties and Mechanism of action of eukaryotic 3-methyladenine-DNA glycosylases. In: Molecular biology of DNA repair. Collins, A., Johnson, R.T. and Boyle, J.M. (eds.). The Company of Biologists Ltd. Cambridge. p.139-147.

Hopwood, D.A. 1970. The isolation of mutants. Methods Microbiol. 3A: 363-433.

Isaac, C.E., Jones, A. and Pickard, M.A. 1990. Production of cyclosporins by *Tolypocladium niveum* strains. Antimicrob. Agents Chemother. 34: 121-127.

Isaac, C.E. 1988. M.Sc Thesis. University of Alberta.

Kay, J.E. 1989. Inhibitory effects of Cyclosporin A on lymphocyte activation. In: Cyclosporin A (Sandimmun®) in autoimmune disorders. In: Cyclosporin-Mode of Action and Clinical Applications. Thomson, H.W. Kluwer Academic Publishers, p.1-24.

Kleinkauf, H. and von Dohren, H. 1987. Biosynthesis of peptide antibiotics. Ann. Rev. Microbiol. 41: 259-289

Kleinkauf, H. and von Dohren, H. 1981. Nucleic acid independent synthesis of peptides. Curr. Top. Microbiol. Immunol. 91: 129-177.

Kleinkauf, H., Roskoski, R. and Lipmann, F. 1971. Pantetheine-linked peptide intermediates in gramicidin S and tyrocidine biosynthesis. Proc. Natl. Acad. Sci.,USA. 68: 2069-2072.

Kobel, H. and Traber, R. 1982. Directed biosynthesis of cyclosporins. Eur. J. Appl. Microbiol. Biotechnol. 19: 237-240.

Krumpiski, V.M., Robbers, J.E. and Floss, H.G. 1976. Physiological study of ergot: Induction of alkaloid synthesis by tryptophan at the enzymatic level. J. Bacteriol. 125:158-165.

Lawen, A. and Zocher, R. 1990. Cyclosporin Synthetase: The most complex peptide synthesizing multienzyme polypeptide so far described. J. Biol. Chem. 265: 11355-11360.

Lawen, A., Traber, R., Geyl, D. and Zocher, R. 1989. Cell-free biosynthesis of new cyclosporins. J. Antibiot. 42: 1283-1289.

Lee, J. and Agathos, S.N. 1991. Dynamics of L-valine in relation to the production of Cyclosporin A by *Tolypocladium inflatum* Appl. Microbiol. Biotechnol. 34: 513-517

Lee, J. and Agathos, S.N. 1989. Effect of amino acids on the production of cyclosporin A by *Tolypocladium inflatum*. Biotechnol. Lett. 11: 77-82.

Madry, N., Zocher, R. and Kleinkauf, H. 1983. Enniatin production by Fusarium oxysporum in chemically Defined media. Eur. J. Appl. Microbiol. Biotechnol. 17: 75-79

Matsumura, M., Imanaka, T., Yoshiomi, T. and Taguchi, H. 1980. Regulation of cephalosporin C production by endogenous methionine in *Cephalosporium acremonium*. J. Ferment. Technol. 58: 205-214.

Merril, C.R., Goldman, D. and van Keuren, M.L. 1984. Gel protein stains: Silver stain. Methods in Enzymol. 104: 441-447.

Moat, A.G. and Foster, J.W. 1988. Microbial Physiology. John Wiley & Sons, Inc. Toronto. p 436-442.

Mody, C.H., Toews, G.B., amd Lipscomb, M.F. 1988. Cyclosporin A inhibits the growth of *Cryptococcus neoformans* in a murine model. Infect. Immun. 56: 7-12.

Peberdy, J.F. 1988. Genetic manipulation. In: Physiology of Industrial Fungi. Berry, D.R. (ed) Blackwell Scientific Publications.London. p187-218.

Queener, S. and Swartz, R. 1979. Penicillins: biosynthetic and semi-synthetic. In: Economic Microbiology. Vol. 3. Secondary products of metabolism. Rose, A.H. (ed) Academic Press. London, New York, San Francisco. p 35-122.

Quay, C., Kline, E.L. and Oxender, D.L. 1975. Role of leucyl-tRNA synthetase in regulation of branced amino acid transport. Proc. Natl. Acad. Sci.(Washington) 72: 3921-3924.

Radman, M. 1977. Inducible pathways in DNA mutagenesis and carcinogenesis. Biochem. Soc. Trans. 5: 903-921.

Rehácek, Z. and De-xiu, Z. 1991. The Biochemistry of Cyclosporin Formation: A Review. Process Biochemistry. 26:157-166

Rolands, R.T. 1983. Industrial fungal genetics and selection. In: The Filamentous Fungi Vol.4 Fungal Technology. Smith, J.E., Berry, D.R. and Kristiansen (eds). p346-372.

Rosen, M.K., Standaert, R.F., Galat, A., Nakatsuka, M. and Schreiber, S.L. 1990. Inhibition of FKBP rotamase activity by immunosuppressant FK506: Twisted amide surrogate. Science. 248: 863-866.

Schendel, P.F., Edington, B.V., McCarthy, J.G. and Todd, M.L. 1983. Repair of alkylation damage. In: Friedberg, E.C. and Bridges, B.A. (eds). Alan R. Liss Inc. p227-240.

Talal, N. 1988. Cyclosporine as an immunosuppressive agent for autoimmune disease: Theoretical concepts and therapeutic strategies. In: Cyclosporine: Applications in autoimmune diseases. Kahan, B.D. (ed).Grune and Stratton, London. p. 11-15.

Traber, R., Hofmann, H. and Kobel, G. 1989. Cyclosporins-new analogues by precursor directed biosynthesis. J. Antibiotics 42: 591-597.

Tropschug, M., Barthelmess, I.B. and Neupert, W. 1989. Sensitivity to cyclosporin A is mediated by cyclophilin in *Neurospora crassa* and *Saccharomyces cerevisiae*. Nature. 342: 953-955.

Vater, J. 1990. Gramicidin S Synthetase. In: Biochemistry of Peptide Antibiotics. Kleinkauf, H. and von Döhren, H. (eds). Walter de Gruyter. Berlin, p. 33-55

von Graffenfried, B., Friend, D., Shand, N., Schies, S. and Timonen, P. 1989. In: Cyclosporin A (Sandimmun®) in autoimmune disorders. In: Cyclosporin-Mode of Action and Clinical Applications. Thomson, H.W. Kluwer Academic Publishers, p.213-252

Weiser, J. and Matha, V. 1988a. The insecticidal activity of cyclosporines on mosquito larvae. J. Invertebr. Pathol. 51:.92-93

Wenger, R.M. 1986. Synthesis of Ciclosporin and Analogues: Structural and Conformational Requirements for Immunosuppressive Activity. In: Progress in Allergy. Borel, J.F. (ed.). Karger, Basel. 38: 46-64

von Arx, J.A. 1986. *Tolypocladium*, a synonym of *Beauveria*. Mycotaxon. 25: 153-158.

Zahner, H. and Kurth, R. 1982. Overproduction of Microbial Metabolites-The supply of precursors from the Intermediary Metabolism. In: Overproduction of Microbial Products.

Krumphanzl, B., Sikyta, B. and Vanek, Z. (eds). Academic Press, New York. p167-179.

Zocher, R., Madry, N., Peeters, H., and Kleinkauf, H. 1984. Biosynthesis of cyclosporin A. Phytochem. 23: 549-551.

Zocher, R., Nihira, T., Paul, E., Madry, N., Peeters, H., Kleinkauf, H., and Keller, U. 1986. Biosynthesis of cyclosporin A: partial purification and properties of a multifunctional enzyme from *Tolypocladium inflatum*. Biochemistry. 25: 550-553.

6. APPENDIX

6.1: Production of Cyclosporin by UV generated mutants.

Mutant #	Final pH	DCW g/L	Cy A mg/L	Cy C mg/L	TOTAL Cy mg/L
1	4.4	5.42	43.61	5.11	48.72
2	5.4	3.52	24.89		24.89
3	5.2	4.50	14.90		14.90
4	5.2	4.70	24.37		24.37
5	5.3	3.36	26.30		26.30
6	5.3	3.22	21.07		21.07
7	5.2	4.10	17.78		17.78
8	5.2	3.94	33.55	1.29	34.84
9	5.1	4.22	25.40		25.40
10	5.2	3.66	28.68	2.88	31.56
11	4.9	3.38	41.20	4.69	45.89
12	5.2	4.46	26.54		26.54
13	5.4	3.16	12.98		12.98
14	5.3	4.56	26.63	4.01	30.64
15	5.3	3.54	13.79		13.79
16	5.4	3.82	24.59		24.59
17	5.4	3.52	16.75		16.75
18	5.3	4.74	46.11	10.07	56.18
19	5.5	4.04	35.53	10.28	45.81
20	5.2	3.24	18.27		18.27
21	5.3	4.94	24.61		24.61
22	5.3	2.70	20.42		20.42
23	5.4	2.66	19.48		19.48
24	5.4	3.06	9.02		9.02
25	5.3	3.72	19.89		19.89
26	5.4	3.22	15.87	1.56	17.43
27	5.3	2.94	6.86		6.86
28	5.2	3.52	19.43		19.43
29	5.4	2.84	18.62		18.62
30	5.4	3.40	17.01		17.01
31	5.3	3.60	30.19	2.48	32.67
32	5.3	3.14	17.36		17.36
33	5.3	4.26	51.16	3.96	55.12
34	5.3	3.48	15.44		15.44
35	5.4	3.54	17.07		17.07
36	4.9	3.00	15.24	1.54	16.76
37	4.9	3.36	103.70	14.70	118.40
38	5.2	2.64	37.41		37.41
39	5.4	2.26	39.24	2.42	41.66
40	5.4	2.70	59.12	4.19	64.84
41	4.2	4.30	81.26	11.07	92.33
42	4.9	2.78	29.64	1.70	31.34
43	4.5	4.60	90.17	12.83	103.00

44	4.4	4.14	61.35	6.35	67.70
45	4.6	4.78	67.72	7.99	75.71
46	4.5	4.98	50.77	6.61	57.38
47	4.2	4.52	99.36	8.93	108.29
48	4.4	4.58	90.11	12.11	102.22
49	5.0	3.72	57.32	9.16	66.48
50	4.4	3.76	63.72	6.42	70.14
51	4.3	4.80	88.09	11.80	99.89
52	5.4	3.20	44.28	4.03	48.31
53	4.2	4.84	49.98	12.50	62.48
54	4.4	3.68	92.61	11.12	103.73
55	4.9	3.40	97.13	18.44	115.57
56	4.7	3.54	55.51	4.65	60.16
57	4.5	4.44	66.51	5.96	72.47
58	5.0	2.44	28.49		28.49
59	5.3	2.72	32.63		32.63
60	4.5	3.86	55.49	5.84	61.33
61	4.5	4.62		1.52	1.52
62	4.4	4.70	61.51	6.00	67.51
63	5.3	2.12	11.25	4.72	15.97
64	4.2	4.20	51.24	8.50	59.74
65	4.7	3.68	67.80	3.48	71.28
66	4.9	2.98	25.18		25.18
67	4.3	3.96	43.58	5.80	49.38
68	5.1	3.40	38.43		38.43
69	4.3	4.38	62.68	4.45	67.13
70	5.4	2.18	19.25		19.25
71	5.8	2.70	38.19		38.19
72	4.8	3.02	68.10	6.13	74.23
73	4.3	4.02	51.89	8.16	60.05
74	4.8	4.06	110.07	17.98	128.05
75	4.4	4.52	70.07	6.32	76.39
76	4.3	4.62	66.80	8.05	74.85
77	4.3	4.38	44.81	14.97	59.78
78	4.3	4.72	57.76	7.42	65.18
79	4.4	4.38	78.07	8.69	86.76
80	4.3	4.90	59.66	4.61	64.27
81	4.7	5.14	47.82	5.21	53.03
82	4.4	5.22	96.91	14.35	111.26
83	4.9	4.18	18.83		18.83
84	5.3	4.00	22.11	2.18	24.29
85	4.3	5.12	87.52	1.61	89.13
86	4.4	4.98	55.55	8.87	64.42
87	4.5	5.16	96.32	17.34	113.66
88	4.4	4.54	86.76	11.10	97.86
89	5.4	2.66	14.86		14.85
90	5.0	5.12	40.83	3.67	44.50
91	5.1	4.44	47.62	3.39	51.01
92	5.2	4.96	24.69		24.69

93	4.3	4.98	95.74	14.18	109.92
94	4.4	5.02	79.14	11.74	90.88
95	4.8	3.98	56.87	4.78	61.65
96	4.3	5.16	96.44	13.30	109.74
97	4.4	4.46	77.03	9.89	86.92
98	4.4	4.78	115.86	18.37	134.23
99	4.4	4.52	79.53	9.94	89.47
100	4.5	4.98	98.86	16.87	115.74
101	4.3	5.02	78.81	10.95	89.76
102	5.0	3.72			0.00
103	4.3	5.14	55.83	7.98	63.81
4002	4.3	5.70	87±2	14±2	101±3

6.2: Cyclosporin production by epichlorohydrin mutants of strain UV-98 generated after 30 minutes of exposure.

Mutant #	Final pH	DCW g/L	Cy A mg/L	Cy C mg/L	Total Cy mg/L
1	4.3	4.84	103.02	30.08	133.10
2	4.2	4.54	80.94	27.37	108.31
3	4.8	3.86	79.53	12.77	92.30
4	4.5	5.76	79.87	19.32	99.10
5	4.9	4.24	54.91	3.24	58.15
6	4.3	4.52	83.78	23.22	107.00
8	4.6	4.34	94.63	22.72	117.35
9	4.6	5.44	84.35	15.96	100.31
10	4.4	3.94	86.67	18.75	105.42
11	4.4	4.46	102.73	24.16	126.89
12	4.3	4.18	101.27	21.19	122.46
13	4.3	4.18	106.95	26.06	133.01
14	4.3	4.48	114.04	32.95	146.99
15	4.4	4.44	103.62	29.07	132.69
16	4.3	4.48	109.74	29.00	138.74
17	4.3	4.52	105.62	24.72	130.34
18	4.3	4.02	83.24	21.22	104.46
19	4.3	5.24	108.38	35.69	144.07
20	4.2	4.74	111.20	31.44	142.64
21	4.4	4.44	89.04	21.02	110.06
22	4.3	4.08	84.75	19.32	104.07
23	4.3	4.28	107.25	29.71	136.96
24	4.2	4.62	92.67	24.33	117.00
25	4.3	4.72	97.79	27.74	125.53
26	4.4	4.20	80.30	18.48	98.78
27	4.3	4.08	87.17	22.08	109.25
28	4.3	4.80	98.45	31.10	129.55
29	4.3	5.25	104.31	22.01	126.32
30	4.4	4.38	91.07	22.48	113.55
31	4.4	4.32	101.59	22.85	124.44
32	4.2	4.46	109.69	29.64	139.33
33	4.5	4.30	87.22	15.83	103.05
34	4.2	4.50	89.27	26.11	115.38
35	4.4	4.44	95.79	21.66	117.45
36	4.2	4.76	84.13	24.26	108.39
37	4.2	5.62	103.64	36.14	139.78
38	4.3	4.48	91.12	17.64	108.76
39	4.3	4.58	109.32	25.84	135.16
40	4.3	4.38	98.55	25.07	123.62

41	4.4	4.66	105.71	25.36	131.07
42	4.3	4.50	106.07	32.97	139.04
43	4.4	5.36	113.47	27.44	140.91
44	4.4	4.52	102.74	29.31	132.05
45	4.4	5.36	107.60	28.75	136.35
46	4.4	4.68	113.32	30.90	144.20
47	4.4	4.42	110.78	25.26	136.04
48	4.4	4.56	106.50	24.62	131.12
49	4.4	4.50	91.77	17.11	108.88
50	4.3	4.90	107.81	30.54	138.35
51	4.4	4.06	89.41	18.68	108.09
52	4.3	3.90	113.70	29.03	142.73
53	4.6	4.36	94.28	18.96	113.24
54	4.4	4.10	123.31	26.00	149.31
55	4.3	3.62	100.17	26.21	126.38
56	4.5	4.56	95.64	17.83	113.47
57	5.8	5.72	69.15	19.47	88.62
58	4.3	4.30	100.69	26.72	127.41
59	4.3	4.00	118.75	27.87	146.62
60	4.4	3.84	104.15	24.85	129.00
61	4.3	4.54	111.86	25.93	137.79
62	4.3	4.24	107.14	27.72	134.86
63	4.4	5.00	94.10	17.63	111.73
64	4.3	3.94	116.24	27.85	144.09
65	4.4	4.22	108.76	21.80	130.56
66	4.4	4.00	100.61	25.72	126.33
67	4.3	3.96	111.73	34.89	146.62
68	4.3	4.46	128.33	35.15	163.48
69	4.3	4.68	115.03	33.15	148.18
70	4.3	4.66	129.02	32.87	161.89
71	4.3	4.74	110.55	30.90	141.45
72	4.3	4.54	119.00	27.23	146.23
73	4.3	4.78	115.57	30.54	146.11
74	4.3	4.74	127.05	33.92	160.97
75	4.3	4.82	109.45	33.74	143.19
76	4.3	5.04	116.96	37.43	154.39
77	4.3	3.82	98.79	28.87	127.66
78	4.3	4.80	133.69	39.79	173.48
UV_98	4.3	3.7±0.3			109±6

6.3: Cyclosporin production by epichlorohydrin generated mutants of UV-98 exposed for 60 minutes.

Mutant #	Final pH	DCW g/L	Cy A mg/L	Cy B mg/L	Cy C mg/L	TOTAL Cy mg/L
1	4.3	3.74	86.73		11.54	98.27
2	4.1	3.80	128.09		17.85	145.94
3	4.2	3.82	82.99		12.81	95.80
4	4.2	3.68	131.91		14.35	146.26
5	4.2	3.76	120.57		11.33	131.90
6	4.2	3.84	121.33		11.60	132.93
7	4.3	3.66	119.09		18.74	137.83
8	4.3	3.58	82.91		11.31	94.22
9	4.2	3.70	134.14		17.85	151.99
10	4.2	3.66	113.87	29.64	13.08	156.59
11	4.2	3.72	83.61	13.81	14.40	111.82
12	4.7	3.90	129.67		16.74	146.41
13	4.4	3.40	91.29		7.19	98.48
14	4.2	3.76	87.06		11.25	98.31
15	4.4	3.64	112.61		13.51	126.12
16	4.3	3.60	82.13	14.21	18.95	115.29
17	4.2	3.42	88.14		11.82	99.96
18	4.8	3.70	150.75	16.02	19.14	185.91
19	4.2	3.50	88.62		12.73	101.35
20	4.2	3.66	84.82		12.81	97.63
21	4.1	3.06	80.49		15.53	96.02
22	4.2	3.60	82.70		15.99	98.69
23	4.2	3.94	84.58		12.28	96.86
24	4.3	3.36	90.07		24.39	114.46
25	4.3	3.60	74.65		11.71	86.36
26	4.2	3.66	124.42		10.77	135.19
27	4.2	2.98	112.26	22.53	29.29	164.08
28	4.5	4.10	75.03		8.78	83.81
29	4.3	3.28	73.55		9.85	83.40
30	4.8	3.74	80.52		13.16	93.68
31	4.2	3.70	88.65		11.06	99.71
32	4.3	4.04	81.30		9.10	90.40
33	4.2	4.32	93.36		12.52	105.88
34	4.2	3.72	78.07		8.67	86.74
35	4.2	3.34	90.91			90.91
36	4.8	5.14	76.51		8.91	85.42
37	4.0	4.36	78.96		8.70	87.66
38	4.2	5.06	78.93		9.05	87.98
39	4.4	4.86	73.87		8.02	81.89
40	4.3	3.36	76.08		6.65	82.73
41	4.2	3.90	83.78		14.32	98.10
42	4.2	3.42	94.22		16.02	110.24
43	4.2	3.20	74.19		8.59	82.78

44	4.2	3.54	74.00		8.96	82.96
45	4.3	3.20	89.59		11.09	100.68
46	4.8	3.76	88.57		9.61	98.18
47	4.0	3.54	140.71	10.77		151.48
48	4.1	3.46	79.76		11.47	91.23
49	4.0	3.46	83.18		8.86	92.04
50	4.8	3.56	75.66		9.42	85.08
51	4.3	3.52	89.16		13.49	102.65
52	4.2	3.28	54.38		5.76	60.14
53	4.7	3.92	90.37		13.24	103.61
54	4.6	3.20	83.43		11.14	94.57
55	4.6	3.46	84.96		15.48	100.44
56	4.9	3.36	65.44		3.88	69.32
57	4.0	3.42	86.87		9.29	96.16
58	4.9	3.46	84.02		22.13	106.15
59	4.1	3.04	92.15		12.49	104.64
60	4.8	3.40	101.60	9.77	19.36	130.73
UV-98	4.4	6.42				79±9